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TITLE: Quantifying the Effects of Preventative Food on the Metabolism of a Prostate Carcinogen in Humans and in Prostate Cells Grown in Culture

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## INTRODUCTION:

**This study is designed to determine primary interventions that will prevent PhIP from causing prostate cancer.** We are investigating the effects of foods associated with reduced prostate cancer risk on a dietary carcinogen known to be associated with cooked meat and elevated cancer risk. Cooked muscle meats, a prominent component of the Western diet, contain potent mutagens and carcinogens belonging to the heterocyclic amine class of compounds. One of these, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) is a genotoxic carcinogen, causing mutations in bacteria [1] and mammalian cells in culture [2]. There have been several animal studies linking PhIP exposure to DNA damage in prostate tissue or prostate tumor formation [3-5]. In humans, prostate tissue has been shown to activate PhIP and DNA adducts have been detected in the tissue after metabolic activation [6].

PhIP is naturally formed in meats during the cooking process, with the highest levels found in grilled or fried meats. There are measurable amounts of PhIP in numerous foods, and in very well-done meats, PhIP can be found at levels up to 400 ng per gram of meat [7]. The human intake of PhIP varies with food type and cooking conditions and is estimated to range from nanograms to tens of micrograms per day [8]. We have developed a method to quantify urinary metabolites of PhIP in human volunteers that have been fed a meal of cooked chicken. This method allows us to understand PhIP metabolism in humans and to measure the effects of potentially chemopreventive foods. At the cellular level we are investigating the metabolism of PhIP in human prostate cancer cells as well as the effect of sulforaphane, the putative active ingredient in broccoli.

### Progress during Year 1:

#### **TASK 1: Determine the stability of PhIP metabolism**

**A) Determine the stability of PhIP metabolism within an individual over time.** Three healthy, normal, male volunteers have been recruited to participate in this phase of the study, which will continue during Year 2. Subjects are asked to abstain from meat consumption for 24 hours prior to being fed a meal that contains 150 g cooked chicken with a known PhIP content. Control urine is collected before eating the chicken and for 4, 6- hour periods (24 hours total) after eating the chicken. Participants are asked to further abstain from cooked meat during the urine collection period. No other dietary restraints are placed upon the individuals. Urine samples are analyzed and metabolites are measured as described in Kulp et.al [9].

A representative LC/MS/MS chromatogram for a human urine sample is shown in Figure 1. We quantify four major human PhIP metabolites: N<sup>2</sup>-OH-PhIP-N<sup>2</sup>-glucuronide, PhIP-N<sup>2</sup>-glucuronide, 4'-PhIP-sulfate, and N<sup>2</sup>-OH-PhIP-N<sup>3</sup>-glucuronide. A discussion of the method development, chromatographic conditions employed, difficulties with the analysis and the results of our first human trial can be found in the manuscript entitled "Identification of urine

metabolites of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine following consumption of a single cooked chicken meal in humans" which can be found in the Appendix of this report.

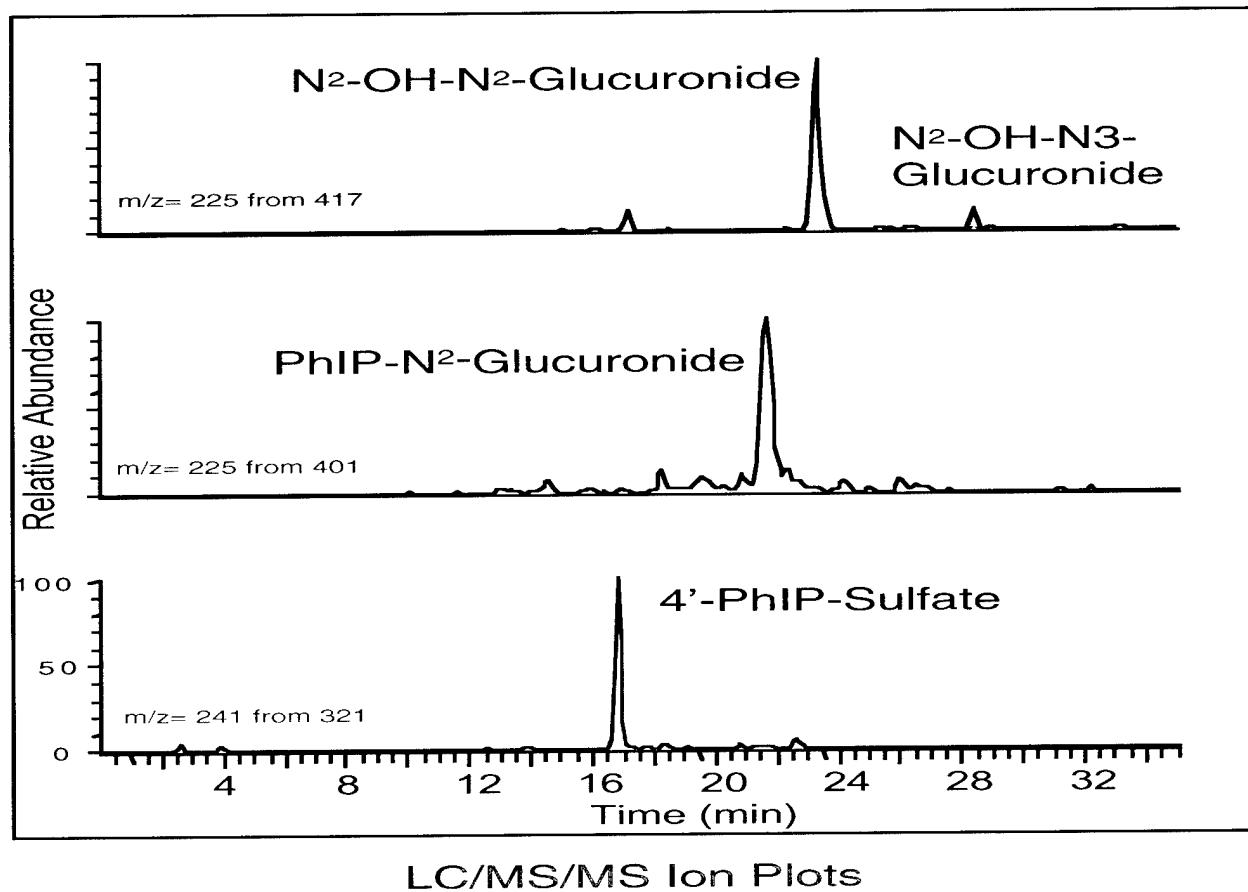


Figure 1. A representative chromatogram of a human urine sample.

At this time, 2 of the volunteers in the metabolism stability study have consumed chicken and collected urine four times: December 1999, April 2000, August 2000 and December 2000 and one of the volunteers has participated 3 times: April 2000, August 2000 and December 2000. The urine collected in December 2000 has been stored, but has not yet been analyzed. The preliminary results from the first three feeding trials are presented in figures 2 and 3.

Figure 2 shows the percent of the total PhIP dose recovered as PhIP metabolites in the urine. Recoveries range from 7-31%, which is consistent with the results of our other human trials. Although these numbers represent only a small fraction of the original dose, recoveries similar to this have been reported in the literature. Two prior studies have quantified PhIP in human urine (by measuring the parent compound and metabolites that have been converted to the parent compound through acid hydrolysis) and in these studies excretion in the urine ranged from 2 to 10% of the ingested dose [10, 11]. Differences in the PhIP metabolites

recovered in the urine may reflect individual variation in digestion and absorption and variation in uptake due to binding of PhIP to other gastrointestinal contents. We are currently pursuing studies that will determine how much of the PhIP present in the meat becomes bioavailable in the digestive tract. These experiments will help us determine if most of the ingested dose that we cannot account for remains undigested and is excreted, which we believe to be the case. Interestingly, in the data presented in figure 1, there appears to be an inverse relationship between PhIP dose consumed and urine recovery; the more PhIP present in the chicken, the less we recovered in the urine. Human intestinal cells contain active transport proteins that have been shown to play a role in PhIP absorption *in vitro* [12]. It is possible that larger PhIP doses saturate these transporters, making absorption less efficient.

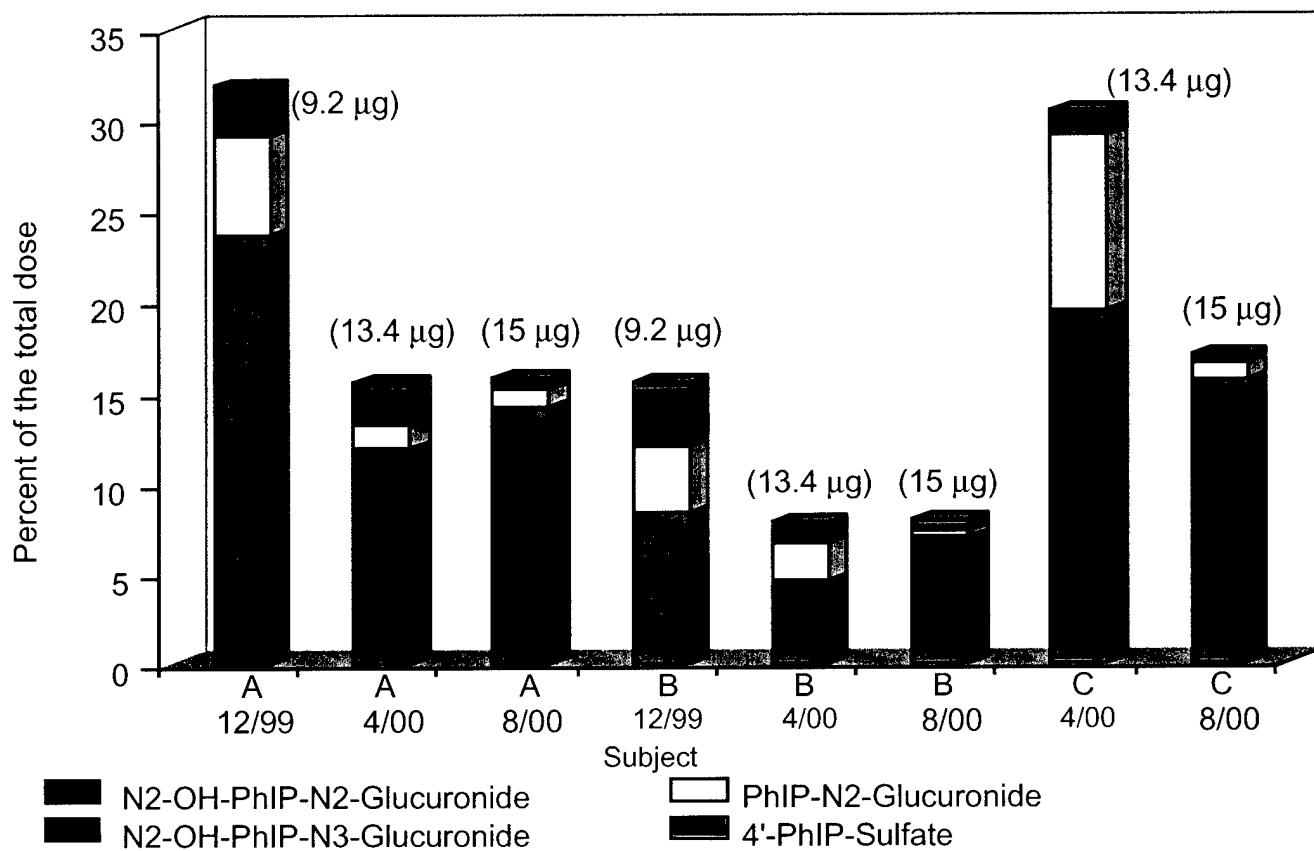


Figure 2. Excretion of PhIP metabolites. Data represent the fraction of the total PhIP dose consumed in the chicken that is recovered as urinary metabolites. Bars are divided into segments that represent the fraction that each metabolite contributes to the total recovered dose. The consumed PhIP dose for each feeding trial is provided in the parentheses.

The bars in figure 2 are divided into segments representing the contribution that each individual metabolite makes to the total recovered dose. In all subjects and in all trials N2-OH-PhIP-N2-glucuronide is the major PhIP metabolite, followed by PhIP-N2-glucuronide. Together these 2 metabolites account for 77-97% of the excreted metabolite. The ratio of metabolites varies both among the individual volunteers and within the same volunteer over time.

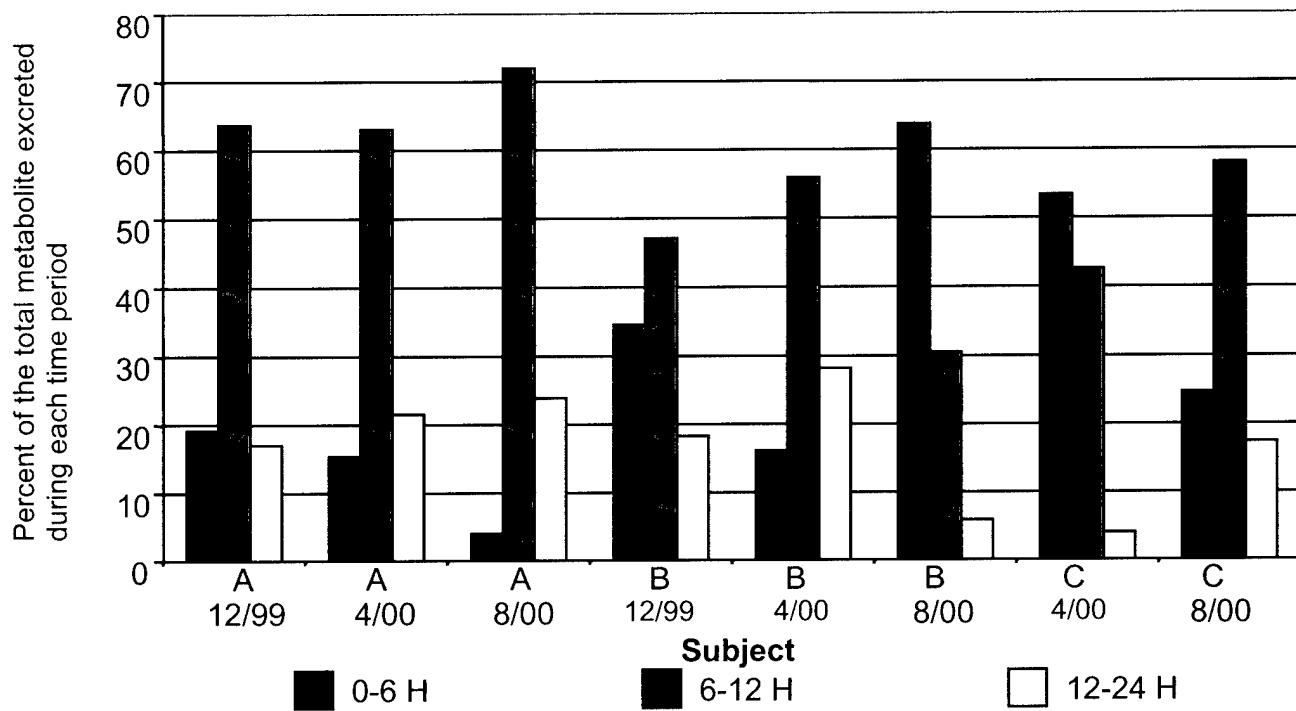


Figure 3. Rate of PhIP metabolite excretion. Each bar represents the percent of metabolite excreted during the given time period.

Because we collect urine in four 6 -hour aliquots, we are able to determine the rate of metabolite excretion (Figure 3). Typically, most of the metabolite is excreted in the 6-12 hour time period, 6 hours after consuming the chicken. Subject A consistently excretes the largest fraction of metabolite during this time. Subjects B & C were not consistent; each had one trial in which they excreted most of the metabolite during the first 6 hours after eating the chicken.

Analysis of the preliminary data presented in Figures 2 and 3 would indicate that there is little consistency in PhIP metabolism within an individual over time. LC/MS/MS analysis of many of these samples will be repeated and the study is ongoing, so no final conclusions can be determined. The enzymes known to be involved in the metabolism of PhIP are found at a variety of levels and activities within the human population [13]. In addition, the activities of these enzymes are changeable and can be effected by diet and lifestyle. Variation in the amounts of PhIP metabolites excreted suggests variation in activity levels of the metabolizing enzymes.

#### **Task 1, B) Determine the assay variability of the same urine sample.**

This task began during the last 6 months of the first year and will extend into year 2. We are doing repeated analysis of one urine sample to determine the stability of the metabolites over time (in urine frozen at -20°C) and the reproducibility of the LC/MS/MS method. The results for three assays of one urine sample are given in Table1.

Table 1. Assay variation for one urine sample. Numbers represent peak area. Each peak area is the average of three injections.

	N2-OH-PhIP-N3-glucuronide	N2-OH-PhIP-N2-glucuronide	PhIP-N2-glucuronide	4'-PhIP-sulfate
15-Sep-00	4044227	103973710	3273079	3795911
03-April-01	14460631	73286162	5606553	5505567
04- April-01	6108774	66487416	5465155	2921107

The average variation in the samples over time is 24-30% for the N2-OH-PhIP-N2-glucuronide, PhIP-N2-glucuronide, and 4'-PhIP-sulfate. N2-OH-PhIP-N3-glucuronide varies 65%. Quantitation has been determined to be a chronic problem with LC/MS response in many laboratories due to changes in the ion path over time. Recognizing the problem, we will run a calibration series of metabolites before sets of urine samples to correct for differences over time. Repeatability of individual injections also varies, ranging from 1 up to 30% coefficient of variation, although most are less than 10%. An external standard was used but was unsatisfactory due to sample carry over. Alternates will be investigated. We will carefully monitor the quantitation variance and seek a solution.

## TASK 2: Human Prostate Cells in culture

### A) Effects of PhIP, N-OH-PhIP and 4'-OH-PhIP on cell proliferation

We have measured the effects of PhIP and a Phase I metabolism intermediate, N-OH-PhIP, on cell growth in the prostate cancer cell lines LNCaP and PC3 and the breast cancer cell lines MCF-7 and MDA-MD-436. The effects of the other Phase I

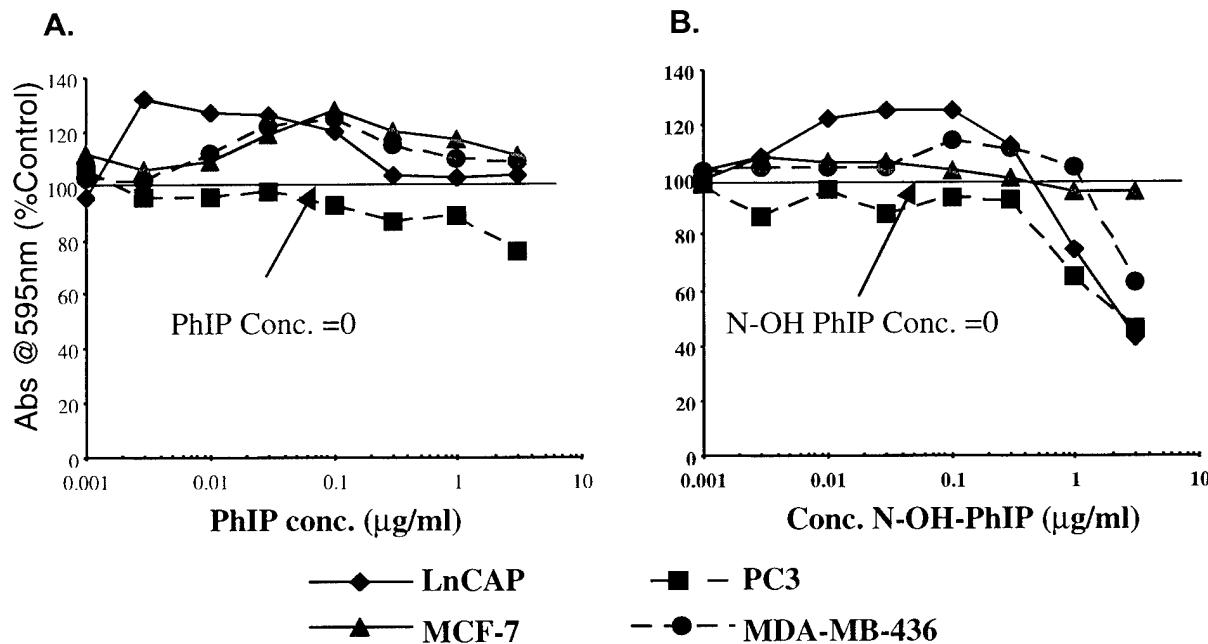


Figure 4. The effects of PhIP (A) and N-OH-PhIP (B) on prostate and breast cancer cell proliferation.

metabolism intermediate, 4'-OH-PhIP, have not been measured at this time. 4'-OH-PhIP is not available commercially and has proven to be more difficult to produce than we expected. We are entirely confident that enough quantities of this compound will be obtained during Year 2 to complete this task.

Figure 4 depicts the effects of PhIP and N-OH-PhIP on prostate and breast cancer cell proliferation. Cell proliferation is assayed with the CellTiter 96 Nonradioactive Cell Proliferation Kit (Pro-Mega) that measures cellular conversion of a tetrazolium salt into a blue formazan product. Cells are plated in 96-well plates and the absorbance of each well is determined spectrophotometrically at 595 nm. Absorbance read is directly proportional to cell number. Breast cancer cells, which are also derived from hormonally responsive tissue, are included for comparison purposes. PhIP consistently stimulates cell growth 20-40% above controls in both of the breast cancer cell lines, as well as the androgen-sensitive prostate cancer cells, LNCaP (panel A). We are in the process of investigating potential mechanisms for this stimulation. Panel B shows the effect of N-OH-PhIP on cell proliferation. In both prostate cancer cell lines and the MDA-MB-436 breast cancer cell line N-OH-PhIP is cytotoxic at concentrations above 0.3  $\mu$ g/ml. The MCF-7 breast cancer cell line is not effected by N-OH-PhIP at the same concentrations, leading us to speculate that 1) the toxic intermediate causing the cell death is not produced in these cells or 2) the cells have a mechanism for detoxification not found in the other cell lines.

### **Task 2, B) Macromolecular binding**

No progress has been made on this task at the present time. Macromolecular binding will be measured in year 2, after we acquire sufficient 4'-OH-PhIP to complete the series of comparative experiments. The 4'-OH-PhIP is being synthesized in the lab of Dr. Mary Tanga at SRI International.

### **Task 2, C) Prostate cell metabolism**

Prostate cell metabolism of PhIP and N-OH-PhIP has been measured by adding these compounds to the cell medium for times up to 48 hours. After incubation metabolites are recovered from the cell medium by solid phase extraction. The cell medium is loaded on a pre-conditioned Oasis® macroporous polymeric column and the metabolites are eluted with methanol. The samples are dried, resuspended in mobile phase and quantified using LC/MS/MS. Columns and conditions used are similar to those used in the analysis of the urine samples. Single ion monitoring MS/MS reveals peaks indicative of PhIP metabolites.

Chromatograms of PhIP-hydroxy- glucuronides extracted from the cell medium from prostate and breast cancer cells are shown in Figure 5. The cells were incubated with N-OH-PhIP for 7 hours before being frozen and thawed three times. Freeze/thaw cycles lyse the cells and free any intracellular metabolites. The medium was extracted as described above and analyzed by LC/MS/MS. Three possible new hydroxy-glucuronides have been tentatively identified (labeled

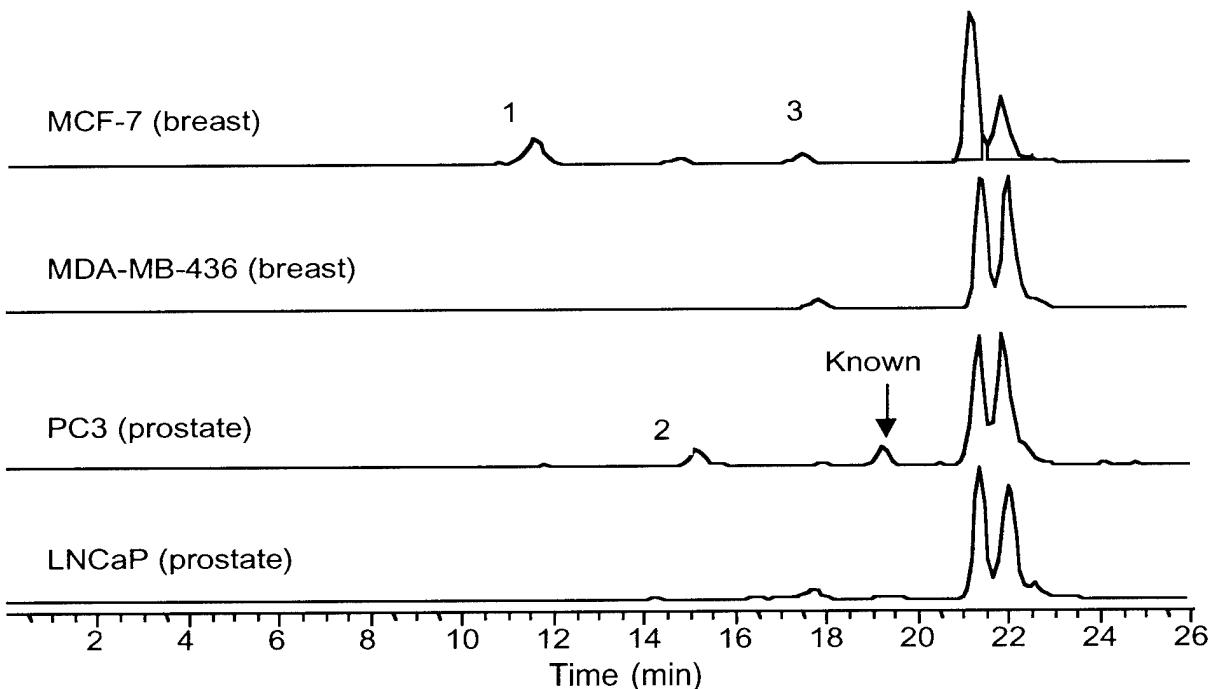


Figure 5. Chromatograms of cell medium from prostate and breast cancer cells. Three possible PhIP -OH-glucuronides (labeled 1,2 and3) and the N2-OH-PhIP-N2-glucuronide metabolite (known) that is also found in the urine are shown.

1,2, and 3 in Figure 5) although their structure is not known. The doublet peak that appears at 21 minutes is a contaminant from the cell medium. Interestingly, there is a metabolite present in the MCF-7 breast cancer cells that is not present in the other 3 cell lines. This is the cell line that is resistant to N-OH-PhIP cytotoxicity. The major metabolite present in human urine, N2-OH-PhIP-N2-glucuronide, is formed in very small amounts in these cells (the peak labeled as "known" in Figure 5). Most of the metabolites found in the urine are formed in the liver. These results may indicate that different pathways metabolize PhIP in the breast and prostate cells as compared to the liver. More work is planned in year 2 to confirm these results and identify the metabolites formed by the cells.

Figure 6 shows ion plots of fragments of mass 401 (mass 225) to detect PhIP-glucuronides. Incubation and extraction conditions are the same as described above. A possible PhIP- glucuronide was tentatively identified in the medium of the prostate cancer cells that was not found in the breast cancer cells. This preliminary result is intriguing as it may indicate a difference in PhIP metabolism between the prostate and the breast. These experiments will be repeated for confirmation in year 2.

By incubating the cells with N-OH-PhIP, the hydroxylated intermediate formed during Phase I metabolism of PhIP, we are primarily investigating Phase II metabolism in these cells. Prostate and breast cancer cells contain active glucuronyl- transferases and sulfotransferases that have the capacity to metabolize steroid hormones. We also examined the medium from these cells for

possible PhIP- sulfate metabolites, but none were detected. N-OH-PhIP, which is hydroxylated on the 2' position, would form 2' OH- sulfates, which are believed to be very unstable. Once we have sufficient quantities of 4'-OH-PhIP, we will repeat these experiments with that Phase I metabolism intermediate, to determine if different metabolites are formed. Sulfates formed at the 4' position are more stable than the 2' position, so it is possible that we will recover PhIP sulfate metabolites from these incubations. In addition, 4'- OH-PhIP is believed to be an intermediate in the detoxification pathway, so any metabolites formed would represent detoxification.

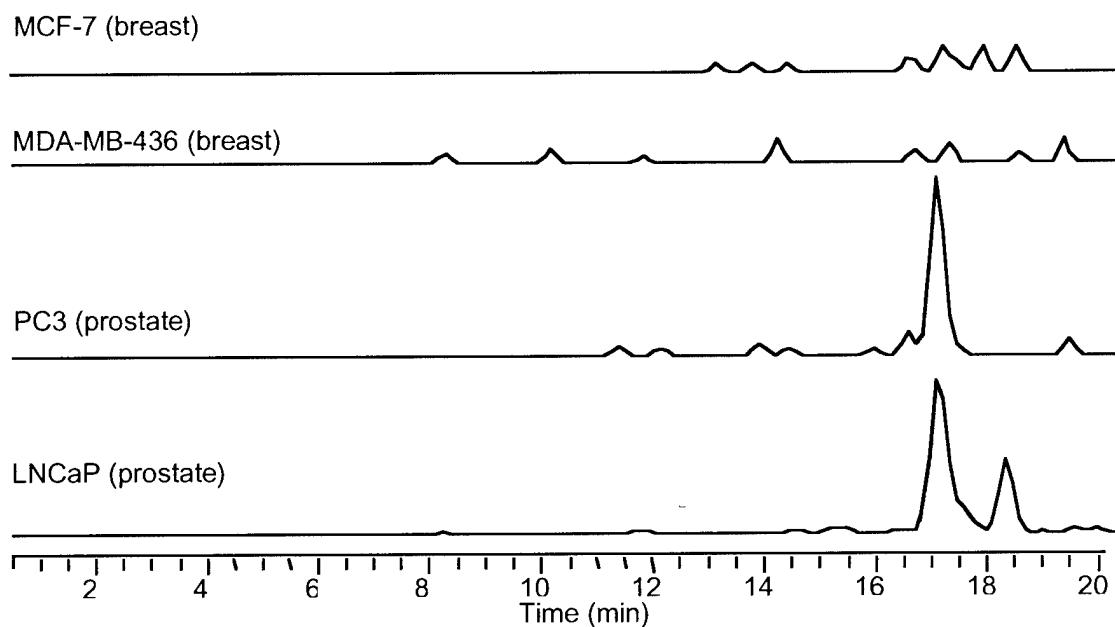


Figure 6. Chromatograms of PhIP- glucuronides from prostate and breast cancer cells.

Prostate and breast cancer cells also contain Phase I metabolizing enzymes, although at very low levels. We have incubated these cells with PhIP to determine if metabolites are formed from the parent compound (representing the product of both Phase I and Phase II metabolism), but none were detected. We will repeat these experiments using increasing concentrations of PhIP, longer incubation times, and better extraction methods. Improved extraction methods will remove more contaminating substances from the cell medium and concentrate any metabolites present at low levels.

### **TASK 3: Link cellular metabolite profiles to urinary metabolite profiles**

This task will best be accomplished after more is known about the metabolites produced by the cells and we have the results of the macromolecular binding experiments. At this time, we have only found one PhIP metabolite that occurs in both the cells and the urine. More work will be done on this task in year 2.

**TASK 4: Chemopreventive interventions**  
**C) Effects of broccoli and sulforaphane**

Although not scheduled until year 3, significant progress has been made on experiments to investigate the effects of broccoli and sulforaphane on PhIP metabolism. Six volunteers have participated in broccoli intervention studies and three of the volunteers have repeated the study during (at 3 month-intervals) year 1.

In humans, we investigated the effect of broccoli on PhIP metabolism by quantifying changes in PhIP urinary metabolites. In this preliminary study, we fed 6 male volunteers well-cooked chicken, collected urine and measured a baseline PhIP urinary metabolite profile. We then gave the subjects one cup of cooked broccoli daily for 3 days. On the fourth day we fed them chicken again and collected urine.

We found that the excretion of the N-hydroxylated PhIP metabolites (the sum of the N2-OH-PhIP-N3-glucuronide and N2-OH-PhIP-N2-glucuronide) changed when compared before and after the broccoli intervention (Figure 7). These preliminary data illustrate that 4 of the individuals (B, D, E, and F) excreted more total N-OH-glucuronide after eating broccoli. Of the 3 individuals who were repeated, 2 increased N-OH-glucuronides in both trials, although the metabolite

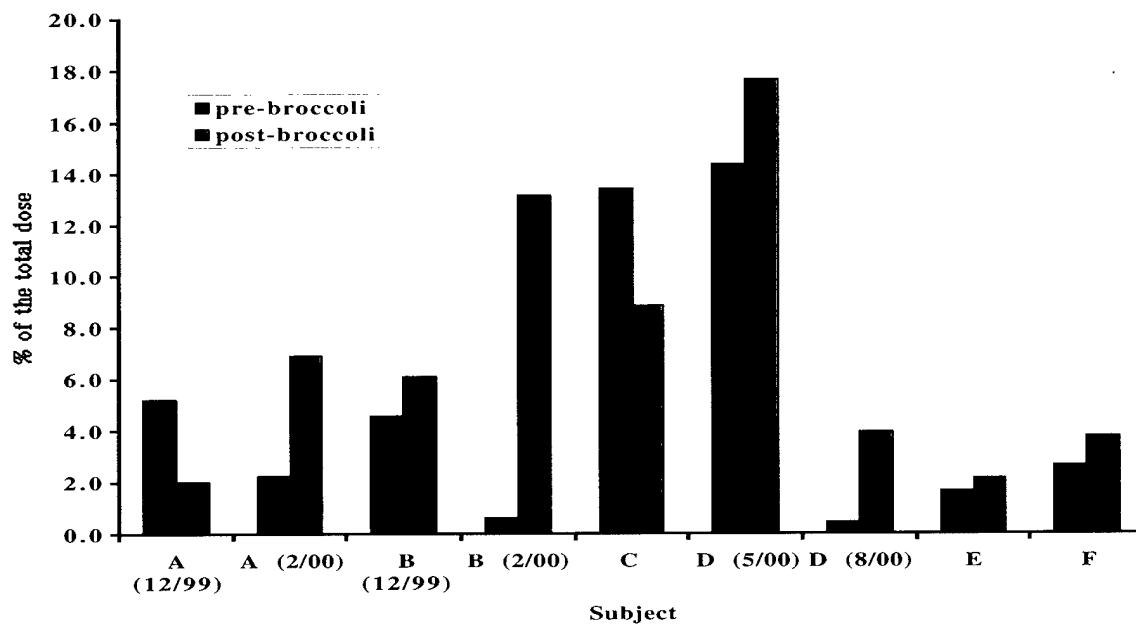


Figure 7. Excretion of N-hydroxylated PhIP metabolites, pre- and post- broccoli intervention.

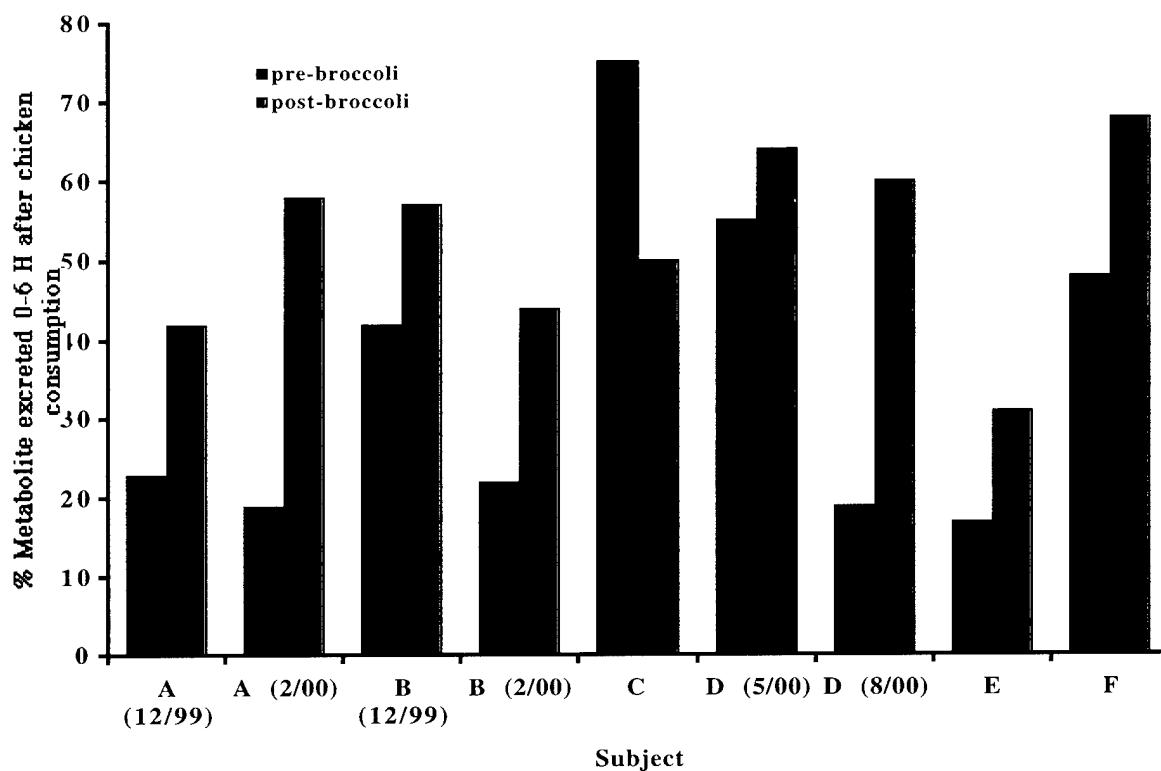


Figure 8. Excretion rate of PhIP metabolites, before and after broccoli intervention.

amounts and magnitude of the effect was much different. Individual A responded differently to the broccoli in the 2 trials, with a decrease in excretion in the 12/99 trial and an increase in the 2/00 trial.

In figure 8, the rate of the excretion of the four PhIP urinary metabolites is shown. These data illustrate that with the exception of volunteer C, more metabolites were excreted in the first 6 hours after the broccoli intervention. Broccoli contains isothiocyanates, which have been shown to induce both cytochrome P450 enzymes and glucuronyl transferases. Our data suggests that broccoli may be affecting both the formation of N-OH-PhIP and the rate of PhIP metabolism, because of the increase in total N-OH-glucuronides and the increase in the fraction of metabolites excreted in the 0-6 hour time period.

At the cellular level, we investigated the effect of sulforaphane on the decrease in prostate cancer cell growth in the presence of N-OH-PhIP. Sulforaphane is one of the isothiocyanates present at high levels in broccoli. It has been shown to be a very potent inducer of Phase II enzyme systems, including glucuronyl transferases [14]. As shown in Figure 9, sulforaphane alone stimulates cell growth 40% above control in PC3 cells and 80% above control in LNCaP cells. The growth- enhancing effect of sulforaphane on cancer cells has not been previously reported. At the concentrations used (1 $\mu$ g/ml), N-OH-PhIP alone

causes a 30% decrease in PC3 cell growth and 20% decrease in LNCaP cell growth. Our hypothesis was that treatment with sulforaphane would induce glucuronyl transferases, thereby increasing the amount of glucuronide metabolites and possibly protecting the cells from the damage caused by N-OH-PhIP. These data do not entirely support this hypothesis. In both cell lines,

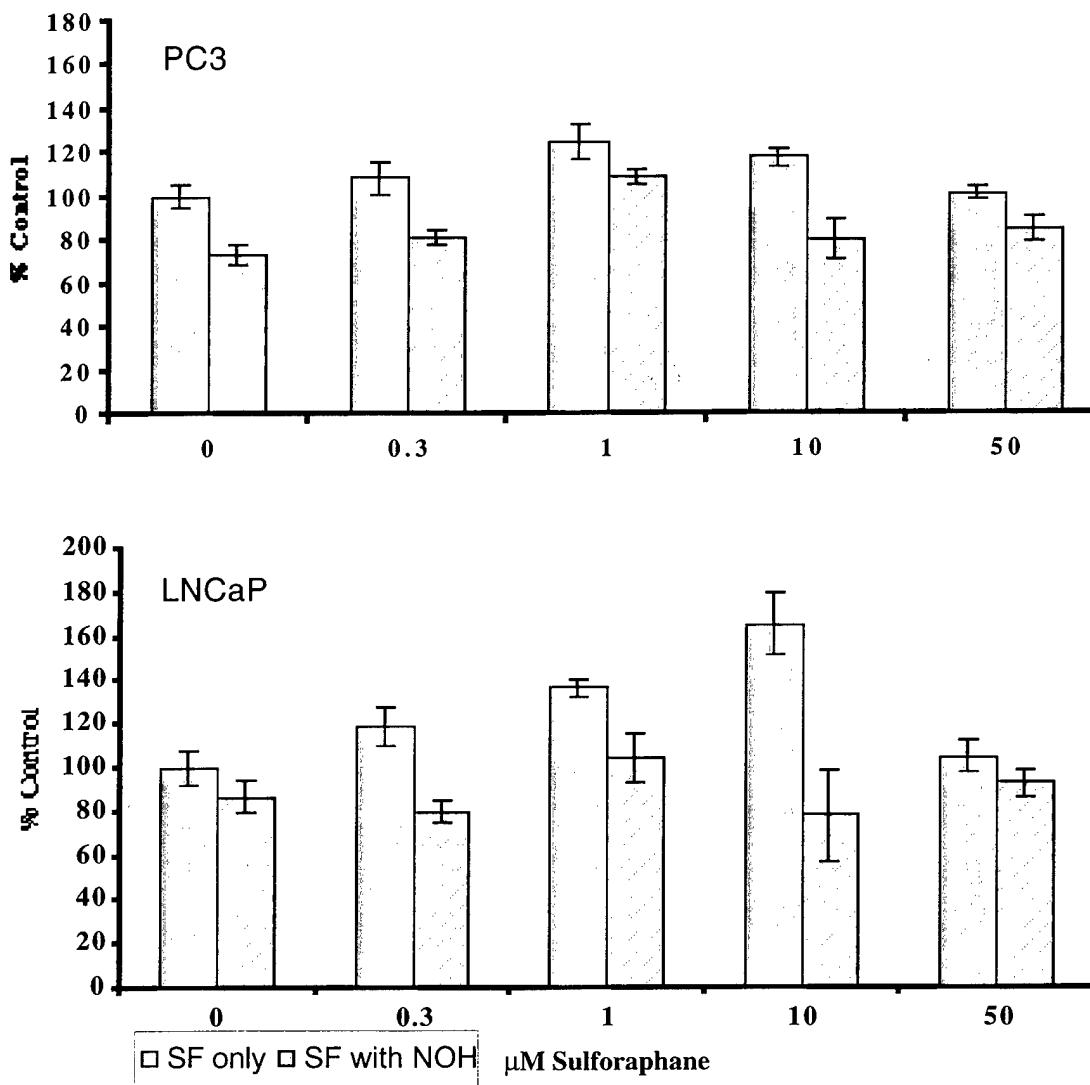


Figure 9. The effect of sulforaphane on the cytotoxic effect of N-OH-PhIP in prostate cancer cell lines.

treating the cells with 1  $\mu$ M sulforaphane abrogated the effect of N-OH-PhIP (cell growth was 100% of control), but in all instances, N-OH-PhIP treatment decreased cell growth compared to sulforaphane alone. These results suggest that 1) sulforaphane does not induce glucuronyl transferases in prostate cells, 2) inducing glucuronyl transferases is not protective against N-OH-PhIP damage or 3) the damage caused by N-OH-PhIP occurs by a mechanism that is unrelated to

sulforaphane treatment. Further experiments will be done to understand these results including measuring PhIP-OH-glucuronide metabolite levels in cells that have been treated with sulforaphane to investigate glucuronyl transferase induction.

#### KEY RESEARCH ACCOMPLISHMENTS:

- ◆ Determined that PhIP and N-OH-PhIP can effect prostate and breast cancer cell growth.
- ◆ Solved problem of short HPLC column life by using laboratory packed columns that can be made inexpensively and the packing discarded frequently.
- ◆ Devised a sample preparation and analysis procedure to identify PhIP metabolites in prostate cancer cells, leading to understanding of metabolism of this prostate carcinogen in the target cells.
- ◆ Determined that broccoli may affect both the rate and relative amounts of PhIP metabolite excretion

#### REPORTABLE OUTCOMES:

Knize, MG, Kulp, KS, Malfatti, MA, Salmon, CP and Felton, JS (2001) " An LC/MS/MS urine analysis method to determine human variation in carcinogen metabolism" J. Chromatography,A. In press.

K.S. Kulp, M.G. Knize, M.A. Malfatti, C.P. Salmon and J.S. Felton, "Detection of human PhIP urinary metabolites following consumption of a cooked chicken meal". UC Davis Cancer Research Symposium; Sacramento, CA; October 2000.

K.S. Kulp, M.G. Knize, M.A. Malfatti, C.P. Salmon and J.S. Felton, "Detection of human PhIP urinary metabolites following consumption of a cooked chicken meal". LLNL Family Days; Livermore CA; September 2000.

K.S. Kulp, M.G. Knize and J.S. Felton "Broccoli affects the metabolism of PhIP in humans", 7th International Conference on Mechanisms of Antimutagenesis and Anticarcinogenesis, Grand Rapids Michigan; Sept. 2000.

K.S. Kulp, M.G. Knize, M.A. Malfatti, C.P. Salmon and J.S. Felton "PhIP metabolites in human urine: Implications for the study of individual variation of carcinogen metabolism and chemoprevention through dietary interactions", LLNL 20/20 , San Ramon, CA, Oct. 2000.

Presentation "Development of Biomarkers for PhIP Metabolism", Department of Community, Occupational and Family Medicine, National University of Singapore, February 28, 2001

Presentation "Heterocyclic Amines: Are they involved in human cancer?" Seminar for Biological Sciences Department, California State University, Stanislaus, March 9, 2001.

Presentation "Factors affecting heterocyclic amine intake and PhIP metabolism in humans", International Conference on Dietary Factors: Cancer Causes and Prevention, Vienna Austria, Feb 16, 2001.

Presentation "Development of Biomarkers for PhIP Metabolism", Department of Community, Occupational and Family Medicine, National University of Singapore, Singapore, February 28, 2001

Presentation "Heterocyclic Amines: Are they involved in human cancer?" Seminar for Biological Sciences Department, California State University, Stanislaus, Turlock, CA, March 9, 2001.

Presentation "Factors affecting heterocyclic amine intake and PhIP metabolism in humans", International Conference on Dietary Factors: Cancer Causes and Prevention, Vienna, Austria, Feb 16, 2001.

Presentation "Do heterocyclic amines cause cancer in humans, and if so what can we do to prevent it?" UC Berkeley Dept of Epidemiology, April 4, 2001.

Presentation "Do heterocyclic amines cause cancer in humans, and if so what can we do to prevent it?" LLNL Biosecurity Facility, December 18, 2000.

Presentation "Do heterocyclic amines cause cancer in humans, and if so what can we do to prevent it?" UC Davis, Cancer Center, October 6, 2000.

Presentation "Do heterocyclic amines cause cancer in humans, and if so what can we do to prevent it?" CSU Deans of Science, November 3, 2000.

Presentation "Do heterocyclic amines cause cancer in humans, and if so what can we do to prevent it?" University of Hawaii, Dept. of Epidemiology, January 18, 2001.

Presentation "Do heterocyclic amines cause cancer in humans, and if so what can we do to prevent it?" University of South Carolina, Cancer Center, November 13, 2000.

Presentation "Do heterocyclic amines cause cancer in humans, and if so what can we do to prevent it?" Children's Hospital, Oakland, CA, Mouse Research Unit, April 27, 2001.

Funding Applied For: NIH Program Project Grant "Determining the carcinogenic significance of heterocyclic amines", pending.

Funding Applied For: California Breast Cancer Research Program "Investigating dietary interactions to prevent breast cancer", pending.

#### CONCLUSIONS:

During the first year of the grant we have initiated a study that will determine the stability of PhIP metabolism within an individual over time, and have investigated the effects of broccoli on PhIP metabolism in humans. We have discovered that PhIP, N-OH-PhIP and sulforaphane have an effect on prostate and breast cell growth and we have devised a sample preparation and analysis procedure to identify PhIP metabolites in prostate cancer cells.

We have had some problems with LC/MS/MS quantitation during the first year. This is not a trivial problem and has been reported to be an issue by many labs. We have solved the problem of short HPLC column life by using laboratory packed columns that can be made inexpensively and discarded when unusable. We are addressing the quantitation issue by re-examining our instrument methods and sample extraction methods.

This research uses state-of-the-art instrument measurement methods to support conclusions about the role of diet and prostate cancer in humans. Although still preliminary, our results indicate that other components of the diet, such as broccoli, may have an effect on the metabolism of a commonly-occurring food carcinogen. Our investigations of the metabolism of N-OH-PhIP and its effects on cell growth in prostate cancer cells may explain why this carcinogen specifically causes tumors of the prostate. It is possible that there are unique metabolic pathways present in prostate cells that produce a reactive intermediate that specifically causes DNA damage in the prostate.

We are on target to continue the work proposed in this grant.

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APPENDICES:

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## Identification of urine metabolites of 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine following consumption of a single cooked chicken meal in humans

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Many studies suggest that mutagenic/carcinogenic chemicals in the diet, like 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), may play a role in human cancer initiation. We have developed a method to quantify PhIP metabolites in human urine and have applied it to samples from female volunteers who had eaten a meal of cooked chicken. For this analysis, urine samples (5 ml) were spiked with a deuterium-labeled internal standard, adsorbed to a macroporous polymeric column and then eluted with methanol. After a solvent exchange to 0.01 M HCl, the urine extracts were passed through a filter, applied to a benzenesulfonic acid column, washed with methanol/acid and eluted with ammonium acetate and concentrated on a C<sub>18</sub> column. The metabolites were eluted from the C<sub>18</sub> column and quantified by LC/MS/MS. In our studies of human PhIP metabolism, eight volunteers were fed 200 g of cooked chicken containing a total of 27 µg PhIP. Urine samples were collected for 24 h after the meal, in 6 h aliquots. Although no metabolites could be found in urine collected from volunteers before eating the chicken, four major human PhIP metabolites, N<sup>2</sup>-OH-PhIP-N<sup>2</sup>-glucuronide, PhIP-N<sup>2</sup>-glucuronide, 4'-PhIP-sulfate and N<sup>2</sup>-OH-PhIP-N<sup>3</sup>-glucuronide, were found in the urine after the chicken meal. The volunteers in the study excreted 4–53% of the ingested PhIP dose in the urine. The rate of metabolite excretion varied among the subjects, however, in all of the subjects the majority of the metabolites were excreted in the first 12 h. Very little metabolite was detected in the urine after 18 h. In humans, N<sup>2</sup>-OH-PhIP-N<sup>2</sup>-glucuronide is the most abundant urinary metabolite, followed by PhIP-N<sup>2</sup>-glucuronide. The variation seen in the total amount, excretion time and metabolite ratios with our method suggests that individual digestion, metabolism and/or other components of the diet may influence the absorption and amounts of metabolic products produced from PhIP.

### Introduction

Cooked muscle meats, major components of the Western diet, contain potent mutagens and rodent carcinogens belonging to the heterocyclic amine class of chemical compounds. Humans are routinely exposed to varying amounts of these food-derived compounds and there is a concern that they may play a role

**Abbreviations:** 4'-hydroxy-PhIP, 2-amino-1-methyl-6-(4'-hydroxy)phenylimidazo[4,5-*b*]pyridine; N-hydroxy-PhIP, 2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine.

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in human carcinogenesis. Of the 19 heterocyclic amines identified, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) is frequently the most mass abundant heterocyclic amine produced during the cooking of beef, pork and chicken (1–5) and in meats purchased in restaurants (6,7).

PhIP is naturally formed in meats during the cooking process, at least in part due to a heat-dependent condensation of creatinine and phenylalanine, two natural components of muscle meats. The highest levels of PhIP can be found in grilled or fried meats. In very well-done flame-grilled chicken PhIP can be found at levels up to 400 ng/g (2). The human intake of PhIP varies with food type and cooking conditions and is estimated to range from nanograms to tens of micrograms per day, depending on individual dietary and cooking preferences (8).

The role of PhIP in cancer initiation has been well established in animals. PhIP has been shown to cause DNA strand breaks, sister chromatid exchanges and form DNA adducts both *in vitro* and *in vivo* (9–14). Short-term exposure to PhIP produces mutations in the large and small intestine of mice (15,16). In rats and mice dose-dependent tumor formation has been consistently demonstrated after PhIP administration and the most common tumor sites appear to be colon, prostate and breast (17–22). Other studies have confirmed the carcinogenicity of PhIP in rodent breast and prostate gland (23,24). In the CDF1 mouse PhIP induces lymphomas, while in the newborn B6C3F1 mouse model it induces adenocarcinomas of the liver (25,26). PhIP exposure can also occur via breast milk: DNA adducts were found in pups that received breast milk from PhIP-exposed lactating rats (27) and increased intestinal tumors were shown in multiple intestinal neoplasia (Min) mice exposed to PhIP via breast milk (28).

In humans, less is known about the potential role of PhIP and related heterocyclic amines in tumor development. Several studies have shown that individuals who eat their meat well done have an elevated risk of cancers at various sites. Zheng *et al.* showed a significant dose-response relation between doneness levels of meat and breast cancer risk, reporting that women who preferred well-done hamburger, steak and bacon have a 4.62-fold greater risk of breast cancer than did women who preferred meats cooked 'rare' or 'medium' (29). Other studies showed increased risk of colorectal adenomas with increased well-done meat consumption (30,31). Lung cancer has also been related to the consumption of fried, well-done meat (32). Other studies, however, have shown either negative or equivocal associations with well-done meat and cancers of the breast (33,34), colon or rectum (35) or prostate gland (1). In all of these studies PhIP and related heterocyclic amine exposure levels are based upon answers to dietary questionnaires. However, the formation of heterocyclic amines is variable and the levels of these compounds found in foods depends on many cooking variables. Dietary surveys have several flaws, including bias, inconsistent reporting and, most importantly, the difficulty in quantifying cooking doneness

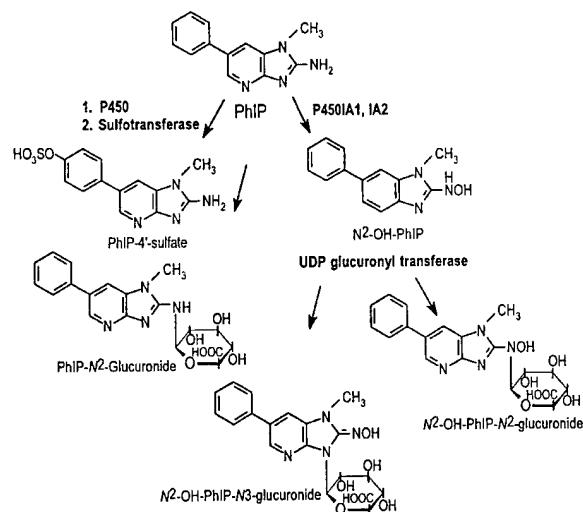


Fig. 1. Major metabolites of PhIP found in human urine.

via questionnaire. As a result, dietary surveys give varying estimates of heterocyclic amine dose that may or may not reflect actual exposures.

PhIP must first be metabolized via Phase I and Phase II enzymes to exert its mutagenic and carcinogenic effect. During Phase I metabolism PhIP is oxidized via cytochrome P4501A2 (CYP1A2) enzymes to a hydroxylated intermediate, 2-hydroxyamino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (*N*-hydroxy-PhIP). *N*-hydroxy-PhIP, which is itself mutagenic, can be converted to a more biologically reactive form via Phase II metabolizing enzymes, primarily the acetyltransferases or sulfotransferases. This esterification generates electrophilic *O*-sulfonyl and *O*-acetyl esters, which have the capacity to bind DNA and cellular proteins (10,36–38). Detoxification primarily involves glucuronidation. *N*-hydroxy-PhIP can form stable glucuronide conjugates at the *N*<sup>2</sup> and *N*<sup>3</sup> positions which can be excreted or transported to extrahepatic tissue for further metabolism (39,40). PhIP can also be hydroxylated at the 4' position, forming 2-amino-1-methyl-6-(4'-hydroxy)phenylimidazo[4,5-*b*]pyridine (4'-hydroxy-PhIP). 4'-Hydroxy-PhIP can be conjugated by sulfation and glucuronidation to polar compounds that are readily excreted (41,42). In addition, the parent compound can be directly glucuronidated at the *N*<sup>2</sup> and *N*<sup>3</sup> positions. These glucuronides are not reactive and this is believed to be a detoxification pathway (40,43). The structure of these metabolites and probable pathways of formation are shown in Figure 1.

Until recently, studies of human PhIP metabolism have been limited to hepatic microsomes or cells in culture. Human hepatic microsomes have been shown to hydroxylate PhIP via CYP1A2 to *N*-hydroxy-PhIP, as well as glucuronidate this intermediate (40,44–46). Other studies have shown that *N*-hydroxy-PhIP can be acetylated by cytosolic hepatic *O*-acetyltransferases (47). In humans, extrahepatic metabolism of PhIP has been demonstrated in breast, prostate and colon. Human mammary cells contain both Phase I and Phase II metabolizing enzymes, giving them the capability to metabolize both PhIP and *N*-hydroxy-PhIP (48–52). *N*-hydroxy-PhIP has been shown to cause DNA damage and produce DNA adducts in human prostate epithelial cells *in vitro*. These cells contain active acetyltransferase enzymes that could activate *N*-hydroxy-

PhIP to the ultimate carcinogenic form (53). Phase II metabolism has also been demonstrated in human colon cytosol (54).

Little has been done to characterize PhIP metabolic pathways in humans, although pioneering work examined the relationship of urinary excretion of the unmetabolized parent compound and the dose received in well-done hamburgers (55,56). Other studies have demonstrated the presence of PhIP and PhIP conjugates in human urine, but in these studies the urine was first treated with acid to hydrolyze the Phase II metabolic conjugates to the parent amine. These investigations proved that PhIP is bioavailable in humans, but did not give information about specific metabolic pathways (57,58). Most recently, specific results about the identity of human PhIP metabolites were obtained in studies that investigated human PhIP metabolism following administration of <sup>14</sup>C-labeled PhIP to patients undergoing cancer surgery (59–61). In these studies body fluids and tissues were examined using accelerator mass spectrometry to investigate human PhIP metabolic pathways.

We recently described human PhIP metabolism in cancer patients receiving a single dose of radiolabeled PhIP. These studies identified four major human PhIP metabolites: *N*<sup>2</sup>-OH-PhIP-*N*<sup>2</sup>-glucuronide, PhIP-*N*<sup>2</sup>-glucuronide, PhIP-4'-sulfate and *N*<sup>2</sup>-OH-PhIP-*N*<sup>3</sup>-glucuronide (60). In the present study we describe a solid phase extraction LC/MS/MS method for quantifying these four metabolites in human urine, following a meal of well-cooked chicken. We applied this method to characterize PhIP metabolism in eight healthy individuals receiving a known dose of naturally produced PhIP.

## Materials and methods

**Synthesis of *N*<sup>2</sup>-OH-[<sup>2</sup>H<sub>5</sub>-phenyl]PhIP-*N*<sup>2</sup>-glucuronide internal standard**  
 The synthesis of deuterium-labeled *N*-OH-PhIP-*N*<sup>2</sup>-glucuronide was carried out in two steps. Pentadeutero-PhIP, labeled on the phenyl ring as previously described (62), was reacted with baculovirus-infected insect cell microsomes expressing human cytochrome P4501A2 (Gentest, Woburn, MA) to produce the *N*-OH-[<sup>2</sup>H<sub>5</sub>-phenyl]PhIP intermediate. Microsomal incubations consisted of 1 mg/ml microsomal protein, 15 mM MgCl<sub>2</sub>, a NADPH regenerating system (1 mM NADP, 15 mM glucose 6-phosphate and 1 U/ml glucose 6-phosphate dehydrogenase) and 100 μM [<sup>2</sup>H<sub>5</sub>-phenyl]PhIP in 0.1 M sodium phosphate buffer, pH 7.4, in a final volume of 0.5 ml. The samples were incubated for 30 min at 37°C. After the incubation time 1 vol of ice-cold methanol was added to the samples to precipitate the proteins. The protein was removed by centrifugation at 5000 r.p.m. for 5 min in a microcentrifuge. The methanolic extracts were concentrated under N<sub>2</sub> and subsequently analyzed by HPLC using a Waters Alliance HPLC system equipped with a 5 μm 4.6×220 mm TSK-Gel ODS-80 TM column (TosoHaas, Montgomeryville, PA). Metabolites were detected using a Waters 990 photodiode array detector. The *N*-OH-[<sup>2</sup>H<sub>5</sub>-phenyl]PhIP was eluted at 1.0 ml/min using a gradient of 30% methanol, 0.1% triethylamine, pH 6, initially to 55% methanol, 0.1% triethylamine, pH 6, over 8 min. The methanol concentration was maintained at 55% from 8 to 20 min. After evaporation of the mobile phase, the yield of *N*-OH-[<sup>2</sup>H<sub>5</sub>-phenyl]PhIP from [<sup>2</sup>H<sub>5</sub>-phenyl]PhIP was ~40%.

Purified *N*-OH-[<sup>2</sup>H<sub>5</sub>-phenyl]PhIP was reacted with microsomes derived from the AHH-1 TK<sup>+</sup>– human lymphoblastoid cell line which expresses human UDP-glucuronosyltransferase 1A1 (Gentest, Woburn, MA). Microsomal incubations consisted of 1.0 mg/ml microsomal protein, 5 mM MgCl<sub>2</sub>, 0.5 mM EDTA, 6.0 mM UDPGA, 60 μg/ml protein alamethicin and 0.1 mM *N*-OH-[<sup>2</sup>H<sub>5</sub>-phenyl]PhIP in 0.1 M Tris-HCl, buffer, pH 7.8, in a final volume of 0.5 ml. Samples were incubated for 6 h at 37°C. After the incubation time 1 vol of ice-cold methanol was added to the samples to precipitate the proteins, as before. The *N*-OH-[<sup>2</sup>H<sub>5</sub>-phenyl]PhIP-*N*<sup>2</sup>-glucuronide was isolated and purified by HPLC using the conditions described above. The identity of the *N*-OH-[<sup>2</sup>H<sub>5</sub>-phenyl]PhIP-*N*<sup>2</sup>-glucuronide was confirmed by co-elution with *N*-OH-PhIP-*N*<sup>2</sup>-glucuronide from rodent urine and LC/MS/MS fragmentation pattern.

## Study design

The study protocol was reviewed and approved by the Institutional Review Board for Human Research at Lawrence Livermore National Laboratory. Informed consent was obtained from each subject prior to beginning the study.

The individuals participating were recruited from the local workforce, were all female, in good health, non-smokers and of normal weight.

#### Meat preparation and controlled dietary period

Boneless, skinless chicken breasts were cut into ~2.5 cm pieces and fried in a non-stick coated pan, sprayed with a vegetable-based non-stick cooking spray, for 25–35 min. Pan temperature was recorded every 5 min, averaging 186°C for the cooking period. At the end of the cooking time the chicken was white with some browning. A representative chicken sample was removed for heterocyclic amine analysis using previously published methods (63). The first two study subjects were provided with 200 g chicken containing 105 p.p.b. PhIP along with other non-meat foods and beverages. The total PhIP dose was 21 µg. The remaining six subjects were given chicken containing 94 p.p.b. PhIP, for a total dose of 18.8 µg.

Subjects were asked to abstain from meat consumption for 24 h prior to eating the well-done chicken breast. There were no other dietary restrictions. Control urine was collected before eating the chicken and for 24 h after in 6 h increments. Samples were refrigerated until analysis. Repeated analysis of these samples over prolonged periods of time (>1 year) have shown no noticeable change in metabolite levels.

#### Extraction of PhIP metabolites

Urine samples (5 ml) were spiked with internal standard (4.2 ng in 5 µl water) and applied to pre-conditioned 60 mg Oasis SPE macroporous polymeric column (Waters, Milford, MA). Metabolites were eluted with 5 ml of methanol. The elution aliquot was evaporated to dryness under nitrogen and the metabolites were redissolved in 2.5 ml of 0.01 M HCl. Proteins and high molecular weight contaminants were removed by filtering the solution through a Centrifuge YM-3 centrifugal filter (Millipore, Bedford, MA). The filtrate was applied to a preconditioned benzenesulfonic acid column (500 mg) (Varian Sample Preparation Products, Harbor City, CA) and the column washed with 6 ml of 10% (v/v) methanol/0.01 M HCl. The metabolites were eluted onto a coupled C<sub>18</sub> column (1000 mg) (Bakerbond SPE; J.T. Baker, Phillipsburg, NJ) with 0.5 M ammonium acetate, pH 8. The C<sub>18</sub> column was washed with 3 ml of 5% (v/v) methanol/H<sub>2</sub>O and eluted from the C<sub>18</sub> column with 50% (v/v) methanol/H<sub>2</sub>O. The metabolites were dried under nitrogen and 1 ml of urine equivalent was injected into the LC/MS in a volume of 20 µl.

Chromatography was done on an Ultra-Plus HPLC system (Microtech, Sunnyvale, CA) equipped with a YMC basic column (3.0×250 mm). Metabolites were eluted at a flow rate of 200 µl/min using a mobile phase of A (water/methanol/acetic acid, 97:2:1) and 5% B (methanol/water/acetic acid, 95:4:1) for 1 min, to 25% B at 5 min, a linear gradient to 100% B at 30 min and held for 5 min.

Analytes were detected with a mass spectrometer (model LCQ; Finnigan, San Jose, CA) in the MS/MS positive ion mode using an electrospray interface. A capillary temperature of 240°C, a source voltage of 4.5 kV and sheath gas of 70 units with no auxiliary gas were used. An ion trap injection time of 1000 ms and one microscan were used.

Alternating scans were used to isolate [M+H]<sup>+</sup> ions at masses 417, 401 and 321 for natural PhIP metabolites and 422 for the pentadecahydro-labeled internal standard metabolite. Collision energy was 25%. Daughter ions were detected at appropriate masses: 241 [M+H-glucuronic acid]<sup>+</sup> and 225 [M<sup>2+</sup>H-glucuronic acid-OH]<sup>+</sup> from 417 for N-hydroxy-N<sup>2</sup>- and N<sup>3</sup>-glucuronide, respectively; 225 [M+H-glucuronic acid]<sup>+</sup> from 401 for PhIP-N<sup>2</sup>-glucuronide; 241 [M+H-SO<sub>3</sub>]<sup>+</sup> from 321 for PhIP-4'-sulfate; 246 [M+H-glucuronic acid]<sup>+</sup> and 230 [M+H-glucuronic acid-OH]<sup>+</sup> from 422 for the internal standard, N-OH-[<sup>2</sup>H<sub>5</sub>-phenyl]PhIP-N<sup>2</sup>-glucuronide.

#### Recovery studies and precision of the assay

The overall recovery of the metabolites was determined by spiking each urine sample with known amounts of N-OH-[<sup>2</sup>H<sub>5</sub>]PhIP-N<sup>2</sup>-glucuronide. Final metabolite amounts were adjusted based on recovery of the internal standard. The effect of the urine matrix on the recovery of metabolites was determined by spiking increasing amounts of the internal standard in 5 ml of water and comparing these recoveries to recovery of the internal standard in 5 ml urine. Ion suppression in the mass spectrometer by co-eluting interference was investigated by spiking human urine extracts with mouse urine containing high levels of the metabolites.

Replicate analyses of several different urine samples were made during the course of the study to determine the precision of the assay.

## Results

#### Method development and urine analysis

The goal of this study was to develop a method that reliably quantifies PhIP metabolites and could be applied to large numbers of urine samples. The initial step of the method

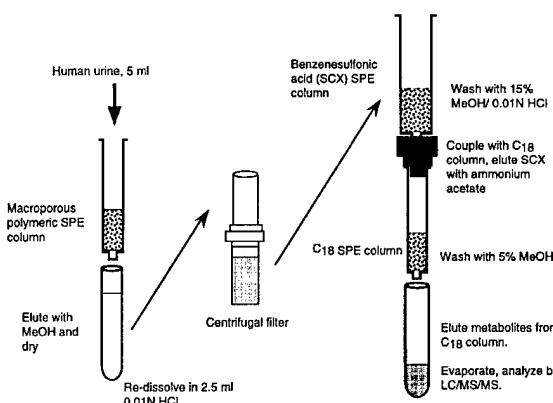
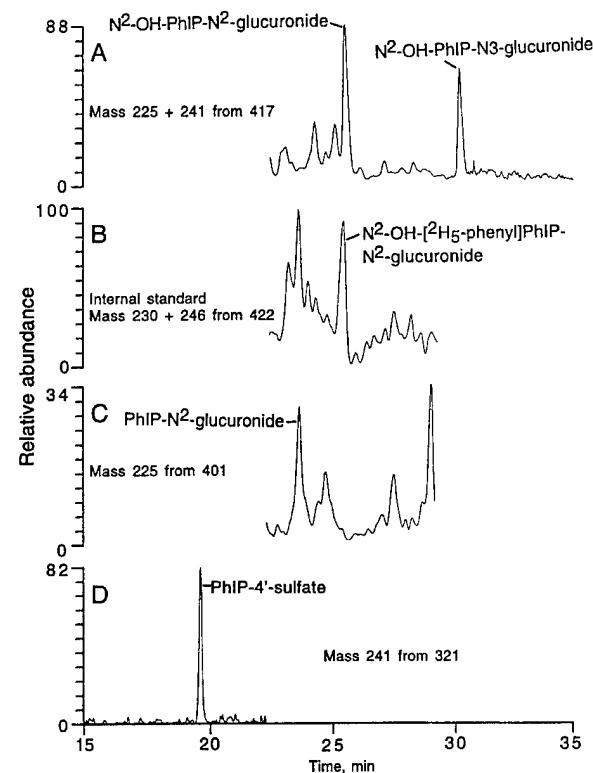


Fig. 2. Urine extraction and concentration scheme.

(Figure 2) utilized non-specific adsorption to remove all the metabolites from the urine, extracting them from the water and salts. Urinary proteins and larger molecular contaminants were removed by centrifuging the extracts through a filter with a molecular weight cut-off of 3000 kDa. Protein determinations of the urine samples before and after filtering demonstrated that 60–80% of the color-reacting material could be removed from the sample during the filtering step (data not shown). This greatly improved HPLC column lifetime. After the initial purification, secondary purifications were designed to exploit the protonation of the heterocyclic nitrogen atoms common to the metabolites in an ion exchange adsorption step removing uncharged interference. Finally, the urine extract was concentrated and washed on reversed phase silica.

Because of the complexity of the urine extracts and the low amounts of metabolite present, metabolites could not be seen by UV or fluorescence detection. Due to co-elution of hundreds of compounds into the mass spectrometer, no signal can be seen above the background with single ion monitoring MS for the parent masses (data not shown). MS/MS detection is necessary for these analyses. Urine samples from rodents receiving high doses of PhIP were used to optimize the HPLC separation and fragmentation of the metabolites. Metabolites in rodent urine were used to determine the linear range of the instrument. The LC/MS/MS peak areas were linear over the range of peaks seen in this study; least squares fits to data gave *r*<sup>2</sup> values ranging from 0.984 to 0.999.

Our method using LC/MS/MS detects peaks for the four identified human PhIP metabolites and the deuterated internal standard in a single chromatographic run (Figure 3). For increased sensitivity, data acquisition was over three segments, isolating mass 321 for 22 min, masses 417, 401 and 422 for 7 min and mass 417 only for the final 5.5 min. Since other ion peaks are often present in the chromatograms that are not one of the four identified PhIP metabolites (Figure 3), expected peak retention times were compared with the internal standard and reference samples to identify PhIP metabolites. N<sup>2</sup>-OH-PhIP-N<sup>2</sup>-glucuronide is typically a broader HPLC peak that fragments into daughter ions with masses of 225 and 241. The sum of these two peak areas is used for quantitation (Figure 3A). The N<sup>2</sup>-OH-PhIP-N<sup>3</sup>-glucuronide is separated in time from the N<sup>2</sup>-OH-PhIP-N<sup>2</sup>-glucuronide and fragments to mass 225 only (Figure 3A). The internal standard, N<sup>2</sup>-OH-[<sup>2</sup>H<sub>5</sub>-phenyl]PhIP-N<sup>2</sup>-glucuronide, elutes slightly before the non-labeled natural product (Figure 3B). The PhIP-N<sup>2</sup>-glucuronide

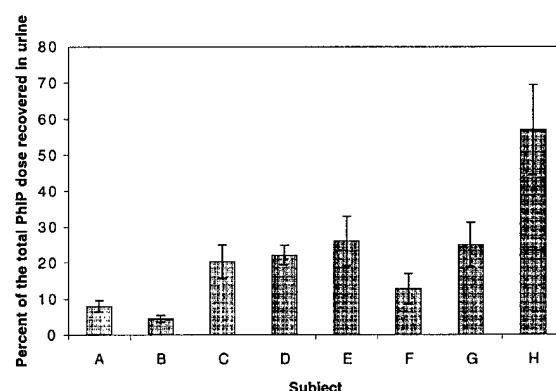


**Fig. 3.** Ion plots of PhIP metabolites and the internal standard from urine of subject H 0–6 h after consuming well-done chicken. A 1 ml equivalent of urine was used. See Materials and methods for LC/MS/MS conditions. (A) Sum of masses 225 and 241 after fragmenting mass 417. These peaks represent  $N^2$ -OH-PhIP- $N^2$ -glucuronide and  $N^2$ -OH-PhIP- $N^3$ -glucuronide. (B) Sum of mass 230 and 246 ions fragmented from mass 422. The indicated peak represents the internal standard. (C) Mass 225 peak plot after fragmenting mass 401, representing PhIP- $N^2$ -glucuronide. (D) Mass 241 ion plot from mass 321. This peak represents PhIP-4'-sulfate.

(mass 401) fragments to mass 225 and typically has the lowest signal-to-noise ratio (Figure 3C). The ion plot of mass 225 from 401 also frequently has peaks at retention times other than the PhIP- $N^2$ -glucuronide (Figure 3C). These additional peaks appear to be specific to individuals and in some cases retention times correspond to PhIP-glucuronides present in urine from animals dosed with PhIP. The PhIP-4'-sulfate (Figure 3D) typically exhibits a sharp peak and good signal-to-noise ratio.

#### Recovery and reproducibility

Spiking human urine samples with animal urine containing high levels of metabolites allowed us to determine the recovery of the metabolites while optimizing the extraction process. This was necessary to ensure that each of the four metabolites was recovered at each step in the clean-up process. Recovery through the final method for each sample was quantified by spiking each urine sample with a deuterium-labeled internal standard,  $N$ -OH-[ $^2$ H<sub>5</sub>]PhIP- $N^2$ -glucuronide. Typical recoveries ranged from 37 to 40% (Table I), although final metabolite amounts in each sample were adjusted based upon recovery of the internal standard in that sample. Recovery of the internal standard is slightly better in water (45–52%), indicating that the complexity of the urine matrix either interferes with the efficiency of the solid phase extraction columns or lowers the



**Fig. 4.** Percent of the dose of PhIP excreted as urinary metabolites for eight volunteers over a 24 h period. The recovery-corrected sum of the amount of  $N^2$ -OH-PhIP- $N^2$ -glucuronide, PhIP- $N^2$ -glucuronide, 4'-PhIP-sulfate and  $N^2$ -OH-PhIP- $N^3$ -glucuronide detected is shown.

**Table I.** Percent recovery of  $N^2$ -OH- $N^2$ -[ $^2$ H<sub>5</sub>]PhIP- $N^2$ -glucuronide spiked into water or urine (numbers in parentheses represent peak area standard deviations of at least two injections of two different samples)

Spike (ng)	Water	Urine
0.5	45.6 ± 8.0	39.8 ± 13.8
1.5	47.3 ± 7.4	40.6 ± 12.3
2.5	52.4 ± 26.4	39.7 ± 5.9

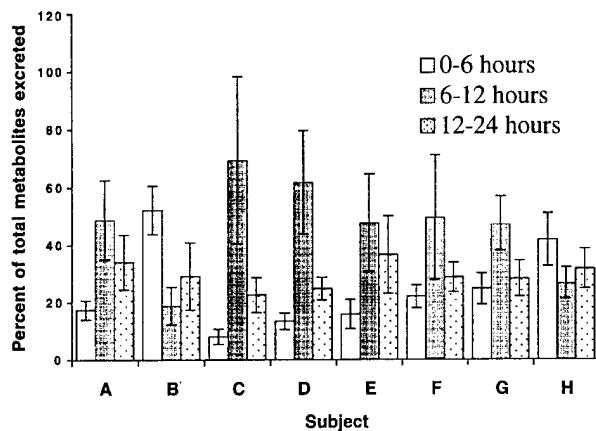
sensitivity of the mass spectrometer through ion suppression. Several of the samples were repeatedly analyzed over the course of the study to determine the reproducibility of the assay. Because of the small peak sizes in our assay, there is variation inherent in the mass spectrometry detection. To account for this variation, each urine extract was injected three times and the peak areas averaged. Variation within a sample ranged from 20 to 30%.

#### Human PhIP metabolite quantitation

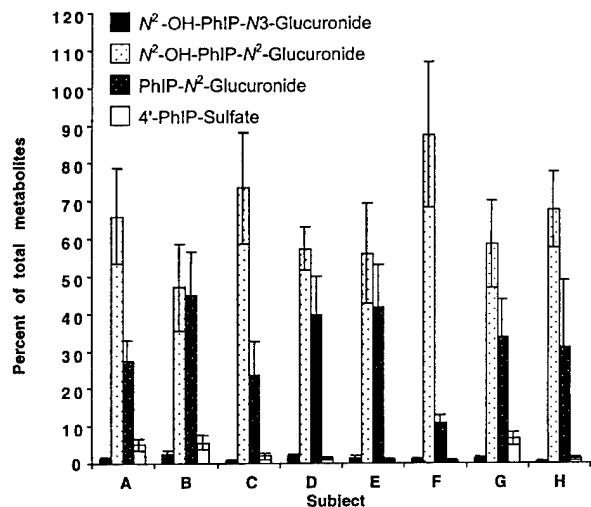
Control urine samples were collected before consuming the well-done chicken, during the period that the volunteers abstained from eating cooked meat. No PhIP metabolite peaks were seen in the control samples from the eight individuals (data not shown). Total urine excreted after chicken consumption was collected for 24 h in 6 h increments. Values shown are corrected for the total volume of urine. Figure 4 shows the percentage of the total dose recovered in urine for the eight subjects. These varied 13-fold, despite the fact that all urine was collected and that each volunteer ate the same amount of chicken.

Figure 5 shows the rate of excretion of the PhIP metabolites in time periods of 0–6, 6–12 and 12–24 h. Three of the individuals (C–E) did not void during the 12–18 h period, thus the data for the 12–18 and 18–24 h periods were combined for the other five subjects. In all of the subjects the majority of the metabolites were excreted in the first 12 h (62–85%). The individuals showed variation in the time of metabolite excretion. Subjects B and H excreted most of the metabolite in the 0–6 h time period (55 and 45% of total metabolites, respectively), whereas subjects C and D excreted the majority in the 6–12 h time period (73 and 69% of total metabolites, respectively).

The total amounts of each of the four individual metabolites



**Fig. 5.** Rate of excretion of four PhIP metabolites in human urine from eight volunteers. Time increments shown are 0–6, 6–12 and 12–24 h after consuming well-done chicken. Total urinary metabolites recovered during the 24 h after dosing were quantified. Data represent the percentage of the total metabolites excreted during the designated time intervals.



**Fig. 6.** Total 24 h excretion of urinary PhIP metabolites for eight individuals. Total excretion of each metabolite during the 24 h collection period was calculated. Data represent the percentage that each individual metabolite represents of the total excreted.

excreted during the 24 h collection period are shown in Figure 6.  $N^2$ -OH-PhIP- $N^2$ -glucuronide is the most abundant urinary metabolite in all individuals.  $N^2$ -PhIP-glucuronide is the second most abundant, but the ratio varies from almost equal amounts of these two metabolites for volunteer B to 8-fold more  $N$ -OH-PhIP- $N^2$ -glucuronide in volunteer F. Together, the  $N^2$ -OH-PhIP- $N^2$ -glucuronide and the PhIP- $N^2$ -glucuronide account for 92–98% of the total metabolite excreted. The PhIP-4'-sulfate and  $N^2$ -OH-PhIP- $N^3$ -glucuronide are present in lower amounts, but also vary among the individual women volunteers.

## Discussion

The opportunity to study a genotoxic dietary carcinogen at realistic levels in humans is rare. PhIP is of special interest because it causes tumors in animals and DNA damage *in vitro* in human tissues. The sites of PhIP damage are among the

most common cancer sites in humans: the breast, colon and prostate gland. In addition, exposure to PhIP need not be ubiquitous, but can be determined and modified through intervention, making PhIP-induced tumor formation preventable.

The metabolism of PhIP has been well characterized in animals, however, little is known about PhIP metabolism in humans. To take advantage of the opportunity to compare animals with humans and humans with each other and see the influence of diet on carcinogen absorption and metabolism, we developed a method for quantifying PhIP metabolites in human urine. This study reports the variation in PhIP metabolism between eight healthy human subjects.

Optimizing a solid phase extraction method was surprisingly difficult due to the polarity of the metabolites. The Oasis brand polymeric absorbant used proved superior to the various brands of  $C_8$  and  $C_{18}$  supports. Because of the wide range in polarity among the metabolites, analyte retention was problematical. To retain as much analyte as possible, washing steps were minimized. Due to the complexity of the urine matrix, there were still co-extracted impurities present in the final sample, even after our extensive clean-up. Our initial attempts at metabolite quantification showed good peak signals and metabolite recoveries, but very poor HPLC column life. Many experiments were done to produce even cleaner samples while maintaining good recoveries. The PhIP-4'-sulfate metabolite was especially problematical because it is more polar than the glucuronide metabolites and thus easily lost during purification. Finally, a satisfactory procedure was devised that gives an acceptable column life of at least 100 injections/column, meeting our goal of devising a method to quantify PhIP metabolites in large numbers of samples.

Well-done chicken is the best source of PhIP exposure because at high temperatures and long cooking times chicken breast preferentially forms more PhIP and less of the related heterocyclic amines as compared with beef. Formation of PhIP seems to be favored by the higher amounts of the amino acids phenylalanine, isoleucine, leucine and tyrosine and lower amounts of glucose that are present in chicken (64). Both the amounts of chicken consumed by our volunteers and the PhIP levels were comparable with consumption levels possible in households or restaurants (7).

The percentage of the dose accounted for in the urine varied among individuals from 4 to 53% and was lower than previous studies of human subjects given radiolabeled PhIP. In the earlier study (60) the subjects were hospitalized elderly cancer patients who were given PhIP in a gelatin capsule. This route of administration resulted in recovery of 90% of the ingested dose in the urine for two of the three subjects. Our study consisted of younger women on their normal diet, which was unrestricted except for refraining from meat consumption for the 24 h prior to dosing. It is probable that the PhIP, when formed in the meat matrix, is not as bioavailable as PhIP in capsule form. In addition, the presence of additional foods in the gastrointestinal tract may influence the absorption of PhIP. We are exploring possible interventions that may reduce PhIP absorption, thereby reducing the biologically available dose.

The kinetics of PhIP metabolite excretion in our study are similar to those seen previously for humans in a study that detected excretion of the parent compound (55). It is also in agreement with our previous study of the excretion of radiolabeled metabolites (60). Our results demonstrate that excretion times vary among the volunteers but that most of

the dose is excreted in the first 18 h. This suggests that these metabolites are suitable for investigating individual variation in rates and ratios of PhIP metabolism. Further, these metabolite measurements may be used as biomarkers of recent exposure, but are not suitable for long-term exposure estimates.

The detection of individual metabolites confirms our earlier findings in cancer patients administered <sup>14</sup>C-labeled PhIP. The major human PhIP metabolites are *N*<sup>2</sup>-OH-PhIP-*N*<sup>2</sup>-glucuronide, PhIP-*N*<sup>2</sup>-glucuronide, PhIP-4'-sulfate and *N*<sup>2</sup>-OH-PhIP-*N*3-glucuronide. As previously reported, human metabolism differs from that seen in rodents. In rodents PhIP-4'-sulfate and 4'-OH-PhIP are the major metabolites, whereas in humans glucuronidation, either directly to PhIP or after *N*-hydroxylation, appears to be a major pathway for urinary excretion. With our assay PhIP activation by cytochrome P450 enzyme-mediated *N*-hydroxylation may be determined, at least in part, by the sum of *N*<sup>2</sup>-OH-PhIP-*N*<sup>2</sup>-glucuronide and *N*<sup>2</sup>-OH-PhIP-*N*3-glucuronide metabolites.

The ratio of the individual metabolites varied among our eight individuals. The enzymes known to be involved in the metabolism of PhIP are found at varying levels and activities within the human population (65). Experiments in animals, in animal tissues grown *in vitro*, in cells grown in culture and in bacteria show that the expression of specific activating enzymes greatly affects the genotoxic response of PhIP. We believe that the *N*<sup>2</sup>-OH-PhIP-*N*<sup>2</sup>-glucuronide and *N*<sup>2</sup>-OH-PhIP-*N*3-glucuronide metabolites represent activation pathways, whereas the PhIP-*N*<sup>2</sup>-glucuronide and PhIP-4'-sulfate represent detoxification pathways. The variation that we can detect in these metabolites suggests that both activation by P450 enzymes and detoxification by UDP-glucuronosyltransferases is variable among individual volunteers and may be an indication of potential susceptibility to DNA damage, mutation and cancer.

Future studies will focus on improving the method by increasing the sensitivity of metabolite detection, allowing us to lower the amount of PhIP-containing meat given to the volunteers. Reducing the analysis time and variation for the LC/MS/MS analysis are also needed. Greater control of the individual's diet before the dosing period may reduce the variation present among the individuals. Both the amount of the metabolite formed and the rate of metabolite excretion can be used to develop a PhIP metabolite phenotype. Repeated analysis of PhIP metabolism in the same individuals over time will help determine the consistency of PhIP metabolism, allowing us to correlate the PhIP metabolite phenotype with genotype.

Altering the metabolism of PhIP to prevent formation of biologically active species may reduce individual susceptibility and prevent the occurrence of cancers in target tissues. Of the metabolites we detected, two appear to be part of the activation pathway for PhIP, *N*<sup>2</sup>-OH-PhIP-*N*<sup>2</sup>-glucuronide and *N*<sup>2</sup>-OH-PhIP-*N*3-glucuronide. It is likely that interventions that reduce *N*-hydroxylation or increase direct glucuronidation of PhIP are desirable, as are genotypes favoring these products. The method described here should make studies of individual susceptibility and dietary interventions possible in the future.

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11      Liquid chromatography–tandem mass spectrometry method of urine  
 12      analysis for determining human variation in carcinogen metabolism

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16

17      **Abstract**

18      We developed a solid-phase extraction LC-MS-MS method for the analysis of the four major metabolites of PhIP  
 19      (2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine) in human urine after a meal of well-done chicken. Ten volunteers each  
 20      ate either 150 or 200 g of well-done chicken breast containing 9–21 µg of PhIP. Among the individual volunteers there is  
 21      8-fold variation in the total amount of metabolites and 20-fold variation in the relative amounts of individual metabolites,  
 22      showing individual differences in carcinogen metabolism. PhIP metabolites were also detected in urine from a subject  
 23      consuming chicken in a restaurant meal, demonstrating the method's sensitivity after real-life exposures. © 2001 Elsevier  
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25      **Keywords:** Amines, heterocyclic aromatic; Aminomethylphenylimidazo [4,5-*b*] pyridine; Pyridines; Glucuronides

26

27      **1. Introduction**

28      PhIP (2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine) is a potent mutagen and rodent carcinogen formed in meats from natural precursors  
 29      during the cooking process. PhIP is found at the highest levels in grilled or fried meats and is frequently the most mass abundant heterocyclic  
 30      amine produced during the cooking of beef, pork, and chicken [1–5], and in meats cooked by professional chefs and purchased in restaurants [6,7]. The  
 31      human intake of PhIP varies with food type and cooking conditions and is estimated to range from

39      nanograms to tens of micrograms per day, depending  
 40      on individual dietary and cooking preferences [8].  
 41      Because humans are routinely exposed to varying  
 42      amounts of these food-derived compounds there is a  
 43      concern that they may play a role in human carcinogenesis.

44      PhIP must first be metabolized via Phase I and  
 45      Phase II enzymes to exert its mutagenic and carcinogenic effect. During Phase I metabolism PhIP is  
 46      oxidized to a hydroxylated intermediate, 2-hydroxy-  
 47      amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (*N*-  
 48      Hydroxy-PhIP). *N*-hydroxy-PhIP is then converted to  
 49      a more biologically reactive form via Phase II  
 50      metabolizing enzymes, primarily the acetyltransferases or sulfotransferases. PhIP can also be hydroxylated at the 4' position, forming 2-amino-1-methyl-6-(4'-hydroxy) phenylimidazo[4,5-*b*]pyridine (4'-hydroxy-PhIP). This hydroxylation does not produce an

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active intermediate. 4'-Hydroxy-PhIP can be conjugated by sulfation and glucuronidation to polar compounds that are readily excreted. Detoxification primarily involves glucuronidation. *N*-Hydroxy-PhIP can form stable glucuronide conjugates at either the *N*<sup>2</sup> or *N*<sup>3</sup> positions. In addition, the parent compound can be directly glucuronidated at the *N*<sup>2</sup> and *N*<sup>3</sup> positions. These glucuronides are not reactive and are excreted in the urine.

There is conclusive evidence that PhIP, a genotoxic carcinogen, is involved in tumorigenesis in animals. In rats and mice, dose-dependent tumor formation has been consistently demonstrated after PhIP administration, and the most common tumor sites in the rat appear to be colon, prostate, and breast [9–14].

Less is known about the role of PhIP in human carcinogenesis. Until recently, studies of human PhIP metabolism have been limited to hepatic microsomes or cells in culture. Pioneering studies in *in vivo* human metabolism demonstrated the presence of PhIP and PhIP conjugates in human urine, but in these studies the urine was first treated with acid to hydrolyze the Phase II conjugates to the parent amine. These investigations proved that PhIP is bioavailable in humans, but did not give information about specific metabolic pathways [15,16]. Specific results about the identity of human PhIP metabolites were obtained in studies that investigated human PhIP metabolism following administration of <sup>14</sup>C-labeled PhIP to patients undergoing cancer surgery. We recently described human PhIP metabolism in cancer patients receiving a single dose of radio-labeled PhIP in a capsule. These studies identified four major human PhIP metabolites: *N*<sup>2</sup>-OH-PhIP-*N*<sup>2</sup>-glucuronide, PhIP-*N*<sup>2</sup>-glucuronide, PhIP-4'-sulfate, and *N*<sup>2</sup>-OH-PhIP-*N*<sup>3</sup>-glucuronide [17].

In the present study we describe our development of a solid-phase extraction LC-MS-MS method for quantifying the four most abundant PhIP metabolites in human urine, following a meal of well-cooked chicken. We applied this method to characterize PhIP metabolism in 10 healthy individuals receiving a known dose of naturally produced PhIP. We have also extended this method to monitor metabolite excretion in a subject consuming chicken as part of a restaurant meal, demonstrating that our method is

sensitive enough to detect PhIP metabolites after common real-life exposures.

## 2. Material and methods

### 2.1. Synthesis of *N*<sup>2</sup>-OH-[<sup>2</sup>H<sub>5</sub>-phenyl]PhIP-*N*<sup>2</sup>-glucuronide internal standard

The biological synthesis of deuterium labeled *N*-OH-PhIP-*N*<sup>2</sup>-glucuronide was carried out in two steps as described previously [18]. Briefly, pentadeutero PhIP was reacted with baculovirus infected insect cell microsomes expressing human cytochrome P4501A2 (Gentest, Woburn, MA, USA) to produce the *N*-OH-[<sup>2</sup>H<sub>5</sub>-phenyl]PhIP intermediate. The reaction products were concentrated under N<sub>2</sub> and then isolated by HPLC using a Waters Alliance HPLC system equipped with a 5 μm, 220×4.6 mm TSK-Gel ODS-80 TM column (TosoHaas, Montgomeryville, PA, USA). Metabolites were detected using a Waters 990 photodiode array detector. The *N*-OH-[<sup>2</sup>H<sub>5</sub>-phenyl]PhIP was eluted at 1.0 ml/min using a gradient starting at 30% aqueous methanol, 0.1% triethylamine, pH 6, to 55% aqueous methanol, 0.1% triethylamine, pH 6, at 8 min. The methanol concentration was maintained at 55% from 8 to 20 min. After evaporation of the mobile phase, the yield of *N*-OH-[<sup>2</sup>H<sub>5</sub>-phenyl]PhIP from [<sup>2</sup>H<sub>5</sub>-phenyl]PhIP was approximately 40%.

Purified *N*-OH-[<sup>2</sup>H<sub>5</sub>-phenyl]PhIP was reacted with microsomes derived from the AHH-1 TK<sup>+/−</sup>-human lymphoblastoid cell line which expresses human UDP-glucuronosyltransferase 1A1 (Gentest). The *N*-OH-[<sup>2</sup>H<sub>5</sub>-phenyl]PhIP-*N*<sup>2</sup>-glucuronide was isolated and purified by HPLC using the conditions described above to give a 15% yield from *N*-OH-[<sup>2</sup>H<sub>5</sub>-phenyl]PhIP.

### 2.2. Study design

The study protocol was reviewed and approved by the Institutional Review Board for Human Research at Lawrence Livermore National Laboratory. Informed consent was obtained from each subject prior to beginning the study. The individuals participating

145 were recruited from the local workforce, were males  
 146 and females aged 22–45 years, in good health, non-  
 147 smokers, and of normal weight.

148 *2.3. Meat preparation and controlled dietary  
 149 period*

150 Boneless, skinless chicken breasts were cut into  
 151 approximately 2.5 cm pieces and fried for 25 to 35  
 152 min in a non-stick coated pan sprayed with a  
 153 vegetable-based cooking spray. Pan temperature  
 154 averaged 186°C for the cooking period. At the end of  
 155 the cooking time the chicken was white with some  
 156 browning. PhIP analysis was performed according to  
 157 previously published methods [19].

158 Subjects were asked to abstain from meat con-  
 159 sumption for 24 h prior to eating the well-done  
 160 chicken breast. There were no other dietary restric-  
 161 tions. The first two study subjects were provided  
 162 with 200 g chicken containing 105 ng/g PhIP. The  
 163 total PhIP dose was 21 µg. Subjects three to eight  
 ✓ 164 were given chicken 200 g of chicken containing 94  
 165 ng/g PhIP, for a total dose of 18.8 µg. The remain-  
 166 ing two subjects were given 150 g of chicken  
 167 containing 62 ng/g PhIP, for a total dose of 9.2 µg.  
 168 All subjects were provided with other non-meat  
 169 foods and beverages with the cooked chicken.

170 Control urine was collected before eating the  
 171 chicken and for 24 h after in 6 h increments.  
 172 Samples were refrigerated until analysis. Repeated  
 173 analysis of these samples over prolonged periods of  
 174 time (greater than 1 year) have shown no noticeable  
 175 change in metabolite levels.

176 *2.4. PhIP metabolite analysis after a restaurant  
 177 meal*

178 To test the sensitivity of detection of this method,  
 179 one subject ordered and consumed chicken that was  
 180 prepared as “chicken mango” at a local restaurant.  
 181 The subject ate approximately 80 g of grilled chicken  
 182 containing 33 ng/g of PhIP (a portion of the entrée  
 183 was reserved and later analyzed using previously  
 184 published methods [19]). Urine was collected for  
 185 approximately 4 h, 4–8 h after eating the meal.

2.5. Extraction of PhIP metabolites

186 Urine samples (5 ml) were spiked with internal  
 187 standard (4.2 ng, in 5 µl water) and applied to a  
 188 pre-conditioned 60 mg Oasis SPE macroporous  
 189 polymeric column (Waters, Milford, MA, USA).  
 190 Metabolites were eluted with 5 ml methanol. The  
 191 elution aliquot was evaporated to dryness under  
 192 nitrogen and the metabolites were re-dissolved in 2.5  
 193 ml 0.01 M HCl. Proteins and high-molecular-mass<sup>+</sup>  
 ✓ 194 contaminants were removed by filtering the solution  
 195 through a Centricon YM-3 centrifugal filter (Milli-  
 196 pore, Bedford, MA, USA). The samples were cen-  
 197 trifuged in the filter at 3000 g, overnight. The filtrate  
 198 was applied to a pre-conditioned benzenesulfonic  
 199 acid column (SCX, 500 mg, Varian, Harbor City, CA,  
 200 USA) and the column washed with 6 ml of 10%  
 201 (v/v) methanol in 0.01 M aqueous HCl. The metabo-  
 202 lites were eluted onto a coupled C<sub>18</sub> column (Baker-  
 203 bond spe, 1000 mg, J.T. Baker, Phillipsburg, NJ,  
 204 USA) with 0.05 M ammonium acetate, pH 8. The  
 205 C<sub>18</sub> column was washed with 3 ml of methanol-  
 206 water (5:95, v/v) and eluted from the C<sub>18</sub> column  
 207 with methanol-water (50:50, v/v). The metabolites  
 208 were dried under nitrogen and 1 ml urine equivalent  
 209 was injected into the LC-MS-MS in a volume of 20  
 210 µl.

211 Chromatography was done on a Microtech Ultra-  
 212 Plus HPLC system (Sunnyvale, CA, USA) equipped  
 213 with a YMC ODS-A column (250×3.0 mm). Metab-  
 214 olites were eluted at a flow-rate of 200 µl/min using  
 215 a mobile phase of A (water-methanol-acetic acid,  
 216 97:2:1) and 5% B (methanol-water-acetic acid,  
 217 95:4:1) for 1 min, to 25% B at 5 min, and a linear  
 218 gradient to 100% B at 30 min and held for 5 min.

219 Analytes were detected with an ion trap mass  
 220 spectrometer (model LCQ, Finnigan, San Jose, CA,  
 221 USA) in the MS-MS positive ion mode using an  
 222 electrospray interface. The capillary temperature was  
 223 240°C and the spray voltage was 4.5 kV. The sheath  
 224 gas was set at 70 units and no auxiliary gases were  
 225 used. The ion trap injection time was 1000 ms and a  
 226 setting of one microscan was used.

227 Alternating scans were used to isolate [M+H]<sup>+</sup>  
 228 ions at mass 417, 401, and 321 for natural PhIP  
 229 metabolites, and 422, for the pentadeutero-labeled  
 230 internal standard metabolite. Collision energy was

233 25%. Daughter ions were detected at appropriate  
 234 masses: 241 [M+H-glucuronic acid]<sup>+</sup> and 225 [M+  
 235 H-glucuronic acid-OH]<sup>+</sup> from 417 for the *N*-hydroxy-*N*<sup>2</sup>  
 236 and *N*<sup>3</sup> glucuronide, respectively, 225  
 237 [M+H-glucuronic acid]<sup>+</sup> from 401 for the PhIP *N*<sup>2</sup>  
 238 glucuronide, 241 [M+H-SO<sub>3</sub>]<sup>+</sup> from 321 for PhIP-  
 239 4'-sulfate, and 246 [M+H-glucuronic acid]<sup>+</sup> and  
 240 230 [M+H-glucuronic acid-OH]<sup>+</sup> from 422 for the  
 241 internal standard, *N*-OH-[<sup>2</sup>H<sub>5</sub>-phenyl]PhIP-*N*<sup>2</sup>-gluc-  
 242 uronide. An external standard of naringenin was used  
 243 in later samples, its [M+H]<sup>+</sup> ion isolated at mass  
 244 273 with protonated fragments detected at mass 147,  
 245 153, and 185.

246 *2.6. Recovery studies and precision of the assay*

247 The overall recovery of the metabolites was  
 248 determined by spiking each urine sample with known  
 249 amounts of *N*-OH-[<sup>2</sup>H<sub>5</sub>]PhIP-*N*<sup>2</sup>-glucuronide. Final  
 250 metabolite amounts were adjusted based on the  
 251 recovery of the internal standard. The effect of the  
 252 urine matrix on the recovery of the metabolites was  
 253 determined by spiking increasing amounts of the  
 254 internal standard in 5 ml of water and comparing  
 255 these recoveries to the recovery of the internal  
 256 standard in 5 ml urine.

257 Ion suppression in the mass spectrometer by co-  
 258 eluting interferences was investigated by spiking  
 259 human urine extracts with mouse urine containing  
 260 high levels of metabolites. In our method, the *N*-OH-  
 261 [<sup>2</sup>H<sub>5</sub>-phenyl]PhIP-*N*<sup>2</sup>-glucuronide is used as a surro-  
 262 gate standard for all of the metabolites because of the  
 263 structural similarity of the metabolites and our belief  
 264 that it is representative of the other metabolites,  
 265 within the precision of other aspects of our assay. An  
 266 external standard of naringenin added to later sam-  
 267 ples shows that ion suppression is consistent and  
 268 suppresses the signal by 65% compared to the  
 269 external standard injected alone.

270 Replicate analyses of several different urine sam-  
 271 ples were made during the course of the study to  
 272 determine the precision of the assay. The coefficient  
 273 of variation was approximately 28% for urine ex-  
 274 tractions and LC-MS-MS, with much of the vari-  
 275 ation occurring in the LC-MS-MS instrument.  
 276 Consequently, samples were injected three times and  
 277 the results averaged.

278 **3. Results and discussion**

279 *3.1. Method development and urine analysis*

280 The goal of this work was to develop a method  
 281 that reliably quantifies PhIP metabolites and could be  
 282 applied to large numbers of urine samples. The  
 283 initial step of the method utilizes non-specific ad-  
 284 sorption to remove all the metabolites from the water  
 285 and salts in the urine. Other materials were tried in  
 286 preliminary work, such as C<sub>4</sub>, C<sub>8</sub>, and C<sub>18</sub> solid-  
 287 phase extraction materials and styrenedivinylbenzene  
 288 medium packed into columns, but none recovered all  
 289 four metabolites as well as the polymeric material in  
 290 the Oasis columns.

291 Our initial attempts at sample clean-up resulted in  
 292 samples that did not chromatograph well. Poor  
 293 HPLC column lifetime, peak broadening, and in-  
 294 creasing retention time for two of the metabolites,  
 295 *N*<sup>2</sup>-OH-PhIP-*N*<sup>2</sup>-glucuronide and PhIP-*N*<sup>2</sup>-glucuro-  
 296 nide were the symptoms of this problem. Suspecting  
 297 that urinary proteins and larger molecule contami-  
 298 nants were the cause of some of these symptoms,  
 299 they were removed by centrifuging the extracts  
 300 through a filter with a molecular mass cut-off of  
 301 3×10<sup>6</sup>. Protein determinations of the urine samples  
 302 before and after filtering demonstrated that 60–80%  
 303 of the color-reacting material could be removed from  
 304 the sample during the filtering step (data not shown).  
 305 This improved HPLC column lifetimes somewhat.  
 306 After the centrifugation step, further purifications  
 307 exploited the protonation of the heterocyclic nitrogen  
 308 atoms that are common to the all the metabolites.  
 309 This ion-exchange adsorption step was designed to  
 310 remove uncharged interferences. Finally, the urine  
 311 extract was concentrated and washed on reversed-  
 312 phase silica.

313 To monitor the recovery of the metabolites  
 314 through the method, a deuterium-labeled internal  
 315 standard is added to the urine before extraction.  
 316 Typical recoveries range from 37 to 40%. Final  
 317 metabolite levels for each sample were adjusted  
 318 based upon the recovery of the internal standard in  
 319 that sample. Because of the small peak sizes in the  
 320 assay, there is variation inherent in the mass spec-  
 321 trometry detection. To account for this variation,  
 322 each urine extract was injected three times and the

324 peak areas averaged. Variation within samples ranged  
325 from 20 to 30%.

326 Because of the complexity of the urine extracts  
327 and the low amounts of metabolite present, metabo-  
328 lites could not be seen by UV or fluorescence  
329 detection. Mass spectrometry must be employed.

330 Urine samples from rodents receiving high doses  
331 of PhIP were used to optimize the HPLC separation  
332 and the fragmentation of the metabolites. Metabolites  
333 in rodent urine were used to determine the linear  
334 range of the instrument. The LC-MS-MS peak  
335 areas were linear over the range of peaks seen in this  
336 study, which is approximately 20-fold higher than  
337 the limit of detection. Internal calibration curves  
338 were calculated for each metabolite based upon  
339 rodent urine spiked into a human urine matrix.  $R^2$   
340 values were:  $N^2$ -OH-PhIP- $N^2$ -glucuronide, 0.9703,  
341 PhIP- $N^2$ -glucuronide, 0.978, PhIP-4'-sulfate, 0.999,  
342 and  $N^2$ -OH-PhIP- $N^3$ -glucuronide, 0.9954.

343 Further, because of the co-elution of hundreds of  
344 compounds into the mass spectrometer, no signal can  
345 be seen above the background with single ion  
346 monitoring MS for the parent masses (Fig. 1A).  
347 MS-MS detection is necessary for these analyses.  
348 Fig. 1B shows a human urine sample analyzed by  
349 LC-MS-MS, showing peaks for the fragments of  
350 four metabolites after the isolation of the parent  
351 masses.

352 Volunteers are asked to refrain from eating meat  
353 for 24 h before eating the cooked chicken, and a  
354 control urine sample is collected at the end of the  
355 meat-free period. A chromatogram that represents a  
356 typical sample of control urine is provided in Fig.  
357 2A. No metabolite peaks are seen at the retention  
358 times of PhIP metabolites. Fig. 2B represents urine  
359 from the same individual, collected during the first 6  
360 h after consuming the chicken. Peaks are clearly seen  
361 for each of the four PhIP metabolites.

362 Fig. 3 shows the percentage that each individual  
363 metabolite represents of the total of all metabolites  
364 excreted over 24 h for 10 individuals. The  $N^2$ -OH-  
365 PhIP- $N^2$ -glucuronide was the major metabolite in all  
366 cases. PhIP- $N^2$ -glucuronide is the second most abun-  
367 dant, but the ratio of these two metabolites varies  
368 from almost equal amounts for subject 2 to 9-fold  
369 more  $N^2$ -OH-PhIP- $N^2$ -glucuronide in subject 6. With  
370 the exception of subject number 10,  $N^2$ -OH-PhIP-

$N^2$ -glucuronide and PhIP- $N^2$ -glucuronide together  
371 account for 90% or greater of the total metabolite  
372 excreted. Subject 10 excreted a much higher propor-  
373 tional amount of  $N^2$ -OH-PhIP- $N^3$ -glucuronide (22%)  
374  $\sim$  3  
375 in contrast to the other individuals, in whom  $N^2$ -OH-  
376 PhIP- $N^3$  glucuronide accounted for 7% or less of the  
377 total metabolite excreted. The time of excretion of  
378 metabolites also varies (data not shown), with some  
379 individuals excreting most of the metabolites in the  
380 0–6 h time period and some later, in the 6–12 h time  
381 period. Little or no metabolite is detected in the  
382 18–24 h time period.

383 To extend our method to real-life exposures, we  
384 collected urine from an individual who had con-  
385 sumed chicken as part of a restaurant meal. Fig. 4  
386 shows the LC-MS-MS chromatogram of a urine  
387 extract collected 4–8 h after consuming the meal.  
388 Peaks for all four metabolites and the deuterium-  
389 labeled internal standard can be detected.

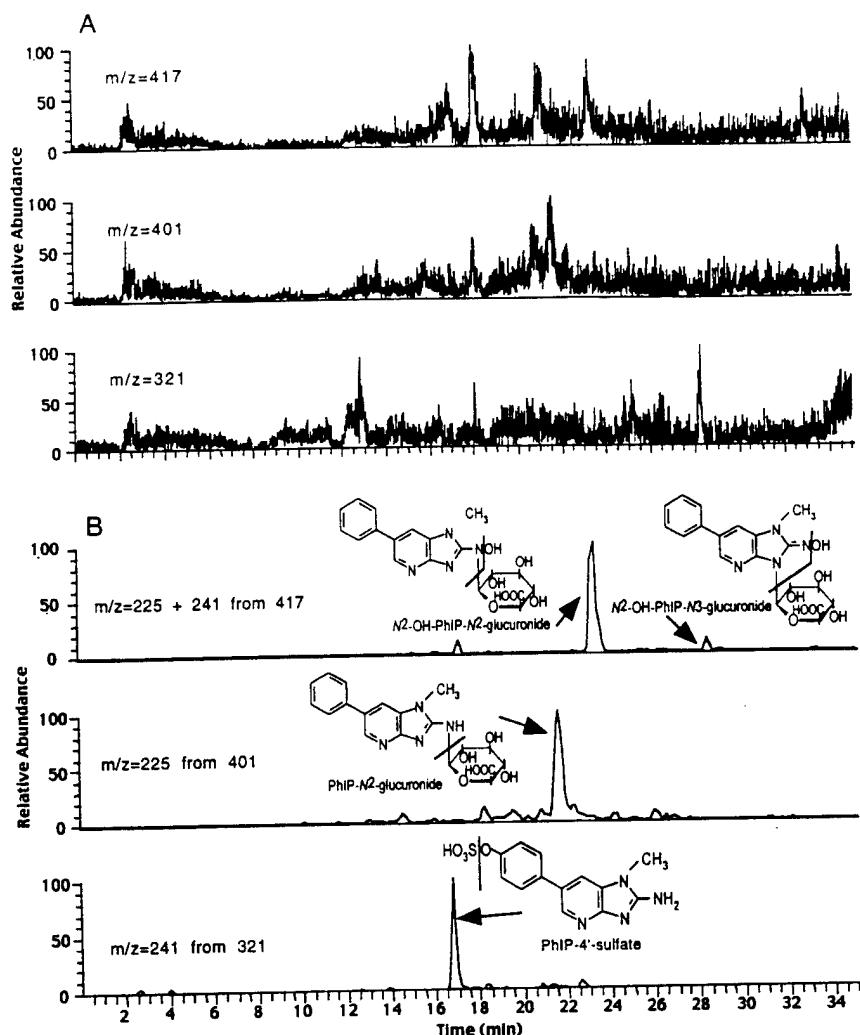
390 Our method provides an opportunity to study a  
391 genotoxic dietary carcinogen at realistic levels in  
392 humans. PhIP is of special interest because it causes  
393 tumors in animals that are among the most common  
394 cancer sites in humans: the breast, colon, and  
395 prostate gland. In addition, exposure to PhIP need  
396 not be ubiquitous, but can be determined and modi-  
397 fied through intervention, making PhIP-induced  
398 tumor formation preventable.

399 Several different types of studies can be supported  
400 by this analysis method. Relative amounts of PhIP  
401 metabolites can be used to determine individual  
402 metabolic phenotype. The effect of diet on car-  
403 cinogen metabolism can be determined by controlled  
404 feeding studies that analyze the changes in the  
405 relative amounts and time of excretion of metabo-  
406 lites. Urine metabolites can also be quantified for  
407 individuals on a normal diet, to monitor for exposure  
408 levels.

409 The enzymes known to be involved in the metabo-  
410 lism of PhIP are found at varying levels and ac-  
411 tivities within the human population [20]. The ex-  
412 pression of specific activating enzymes has a great  
413 affect on the biological reactivity of PhIP. We believe  
414 that the  $N^2$ -OH-PhIP- $N^2$ -glucuronide and  $N^2$ -OH-  
415 PhIP- $N^3$ -glucuronide metabolites represent the meta-  
416 bolic products of activation pathways, whereas the  
417 PhIP- $N^2$ -glucuronide and 4'-PhIP-sulfate represent

✓ N3  
✓ N4  
✓ N5

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421 Fig. 1. Reversed-phase HPLC mass chromatograms of urine extract. (A) shows full scan plot of the  $m/z$  417, 401 and 321 corresponding to  
 422 PhIP metabolites. (B) MS-MS chromatograms of the human urine sample with masses isolated as indicated. Peaks are clearly seen for four  
 423 metabolites indicated by arrows. Chemical structures and a line indicating the site of fragmentation for each structure are shown.

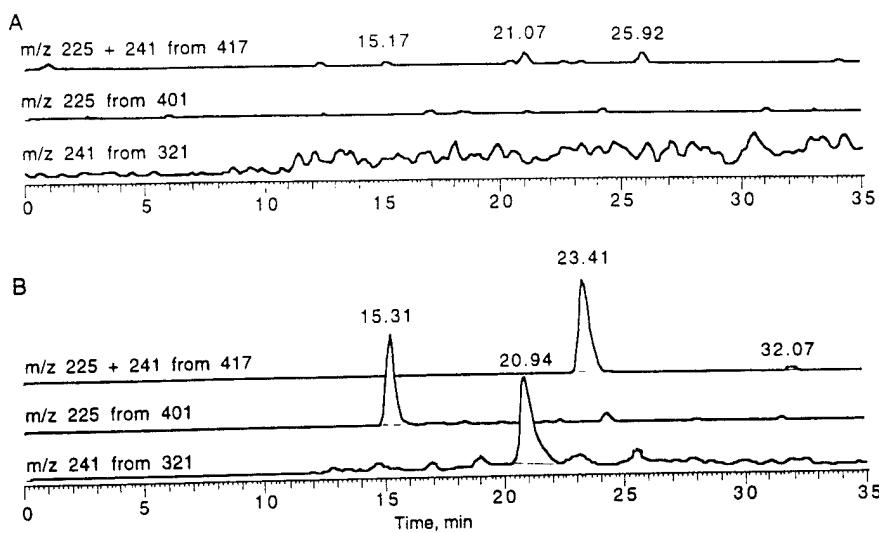
424 detoxification pathways. The variation that we detect  
 425 in these metabolites suggests that the levels of both  
 426 activation and detoxification enzymes varies among  
 427 individual volunteers and may be a way to quantify  
 428 individual phenotype or genotype. Using our method  
 429 to generate a metabolic profile could provide an  
 430 indication of potential susceptibility to DNA dam-  
 431 age, mutation, and cancer.

432 On possible mechanism for the protective effects  
 433 of fruits and vegetables seen in human cancer studies

434 is the influence of natural compounds on both  
 435 primary and secondary metabolism. This suggests  
 436 that the metabolism of carcinogens, including PhIP,  
 437 can be modified by the addition of protective foods  
 438 to the diet. Our method provides an invaluable tool  
 439 for monitoring the effect dietary interactions on PhIP  
 440 metabolism. These effects on metabolism can be  
 441 quantified in humans at normal dietary levels using  
 442 our method.

443 Determining the dietary dose of PhIP is important

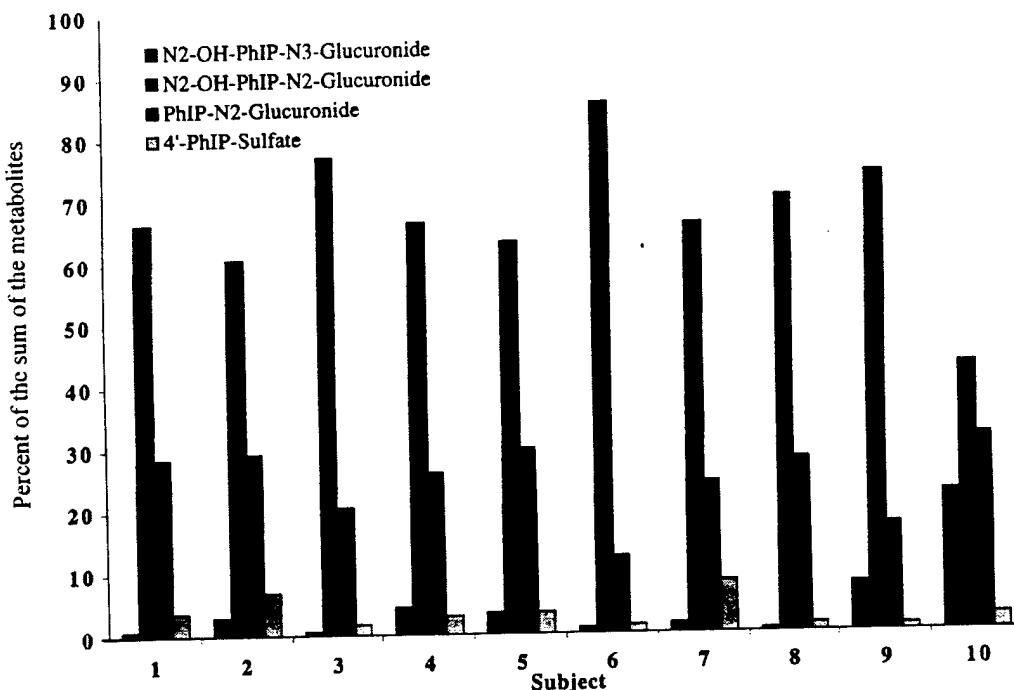
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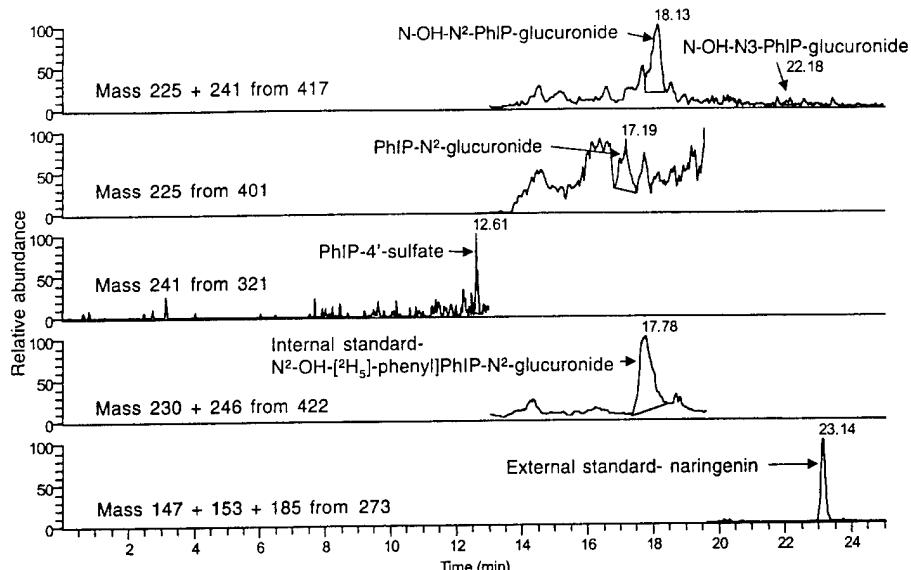
447 Fig. 2. LC-MS-MS chromatograms of urine from a subject fasting from well-done meat for 24 h (A), and urine collected 0–6 h after  
 448 consumption of well-done chicken (B).

*obstaining*



450

451 Fig. 3. Graph of individual PhIP metabolites excreted over 24 h from 10 individuals eating a single meal of well-done chicken.



454

455 Fig. 4. LC-MS-MS mass chromatograms of urine collected after consumption of a restaurant meal of grilled chicken. Peaks identified are  
 456 at the retention time of metabolites or the added internal or external standard. The equivalent of 2 ml of urine and 5 ng of internal standard  
 457 were injected.

458 for epidemiology studies and risk determination.  
 459 Typically, exposure estimations are made through  
 460 dietary questionnaires. However, the formation of  
 461 PhIP is variable, and the amount in foods depends on  
 462 the cooking methods. Dietary surveys have several  
 463 flaws, including bias, inconsistent reporting, and  
 464 most importantly, the difficulty in quantifying cook-  
 465 ing doneness via questionnaire. As a result, dietary  
 466 surveys give varying estimates of PhIP amounts that  
 467 may or may not reflect actual exposures. PhIP  
 468 metabolite detection in the urine of the subject who  
 469 ate chicken prepared at a restaurant demonstrates that  
 470 our method is sensitive enough to monitor PhIP  
 471 exposure of individuals in real-life situations.

472 Future studies will focus on improving the method  
 473 by increasing the sensitivity of metabolite detection,  
 474 allowing us to lower the amount of food containing  
 475 PhIP given to the volunteers. Reducing the analysis  
 476 time and variation for the LC-MS-MS analysis are  
 477 also needed. Repeated analysis of PhIP metabolism  
 478 in the same individuals over time will help determine  
 479 the consistency of PhIP metabolism, allowing us to  
 480 correlate the PhIP metabolite phenotype with geno-  
 481 type.

482 Altering the metabolism of PhIP to prevent forma-

483 tion of biologically active species may reduce in-  
 484 dividual susceptibility and prevent the occurrence of  
 485 cancers in target tissues. The method described here  
 486 should make studies of individual susceptibility and  
 487 dietary interventions possible in the future.

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 492 grant CA55861. 17-

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